Evaluation and comparison of African swine fever virus and vaccinia virus cell entry pathways

by

Matthew Evan Olcha

D.V.M., Kansas State University, 2017

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
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Abstract

African swine fever virus (ASFV) causes a highly contagious and deadly disease in swine, with severe economic consequences. In the absence of a vaccine, control measures that target ASFV replication steps are being actively pursued. Due to the high risk of ASFV and its foreign animal disease status, in the United States it is regulated as a select agent and requires BSL-3 containment. In addition, virulent strains of ASFV require primary cells for propagation in tissue culture. In the face of these limitations the ability to use less restrictive surrogate viruses is attractive. One such option is Vaccinia virus (VV), a member of family *Poxviridae*. Though VV has been used in this capacity before, investigation into its use in Vero cells are limited. When examined using Vero cells treated with various chemical inhibitors, VV produced similar results as the Vero cell adapted strain of ASFV, suggesting the two viruses behave similar during cell entry and early infection. VV provides a suitable surrogate virus for ASFV research, as well as a virus that can replicate in both Vero cells and primary swine macrophages, though there are limited studies regarding the latter.

Swine macrophages and monocytes are the primary target cells for ASFV infection, though factors responsible for this tropism are unknown. A significant body of work identified CD163 as required for ASFV cell entry; however, macrophages lacking CD163 and CD163 knockout pigs support infection. ASFV also utilizes macropinocytosis, a non-specific cellular uptake pathway, to enter the cell. This may explain why CD163KO pigs can be infected with ASFV. The present data indicates that CD163 is not required for ASFV infection, but does not rule out its involvement entirely.

Macropinocytosis inhibitors used in all experiments included EIPA, cytochalasin D, and wortmannin. Inhibitors of clathrin-mediated endocytosis included chlorpromazine and dynasore.

ML-7 inhibits myosin light chain kinase and nocodazole inhibits microtubule dynamics. All seven inhibitors reduced the infection rate, consistent with ASFV using both macropinocytosis and clathrin- and dynamin-dependent endocytosis, as well as non-muscle myosin II and microtubules during stages of entry and early infection. However, the inhibitor effects were not significantly different between wildtype and CD163KO macrophages, suggesting that CD163 lacks involvement with ASFV infection. This work provides a framework for VV as an early infection surrogate model in Vero cells, and helps close the door on the CD163 controversy.

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Table of Contents

List of Figures

List of Tables

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Dedication

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Chapter 1 - Overview of African swine fever and current challenges

Introduction

Pork is big business in the United States, with pork exports in 2018 exceeding 5 billion pounds (USDA, 2019b). A foreign animal disease event, such as the introduction of African swine fever virus (ASFV) would greatly impact this number.

African swine fever was first described in Kenya in 1921 following introduction of the domestic pig into the country in 1910. Early transmission studies conducted by Montgomery in 1914 and 1916 found that wild African suids such as warthogs (*Phacochoerus africanus*) can become infected and harbor virus in blood without showing clinical disease. These studies also showed that transmission can occur between domestic pigs via excretions such as urine and feces. Virus in biological materials is persistent in the environment, with naïve pigs becoming infected when placed in a pen that was not cleaned after housing infected animals. While ASFV spreads readily between domestic pigs via direct contact, this does not seem to be the case between warthogs, and from warthogs to domestic pigs (Eustace Montgomery, 1921). It was determined that vector borne transmission by soft ticks is crucial for maintenance of the virus in warthog populations, as well as a potential route of spillover into domestic pig populations (Parker et al., 1969). In sub-Saharan Africa, the sylvatic transmission cycle between warthogs and soft ticks of the genus *Ornithodoros* generally goes unnoticed. These nidicolous ticks inhabit warthog burrows and can transmit the virus to juvenile warthogs, which develop a transient viremia. In ten surveyed areas throughout southern and eastern Africa where tick vectors are present, greater than 80% of the sampled warthog population tested positive for antibodies to ASFV (Plowright W., Thomson G. R. et al., 1994). A domestic pig-tick cycle has been described in areas where soft ticks are present, such as Malawi, Portugal, and Spain. However, in other regions such as west Africa, the role of soft ticks has not been described. The disease remained

confined to Africa until transcontinental spread of contaminated waste brought the disease to Portugal in 1957. This situation was controlled until a second incursion in 1960 started an epidemic which lasted over 30 years (Costard et al., 2013). By 1985 ASF had entered France, Italy, Belgium, the Netherlands, Brazil, Cuba, the Dominican Republic, and Haiti (Arias et al., 2008). Control measures were successful, though extreme. For example, the island of Hispaniola was completely depopulated of swine. In Haiti alone almost 400,000 pigs were slaughtered during a campaign costing upwards of 15 million USD (Alexander, 1992). Arias et al recently reassessed the eradication effort Spain launched in 1985. Spain's coordinated plan introduced mobile veterinary teams, serologic surveillance of all pig farms, improvements in animal holding facilities, depopulation of infected herds, and strict regulation involving animal movement. Protection and surveillance zones were established in response to an outbreak, and about one month following a herd depopulation affected buildings were completely disinfected and all animal products and feed destroyed. Farms were not repopulated until healthy sentinel animals introduced to the premises remained serologically negative. While this program was successful it involved many years of continued effort, a large budget, and producer compliance. As progress was made, establishing regions (ASF-free region, ASF infected-region, Surveillance region) allowed trade prohibitions to be gradually lifted (Arias et al., 2008). Two lessons from the Spanish model are the necessity of farmer education and cooperation, and the importance of establishing defined zones to minimize impact on trade. France, Italy, Belgium, and the Netherlands were also successful at eradicating the disease, with the exception of the island of Sardinia where the disease remains endemic. Brazil implemented a similar emergency plan that led to eradication of the disease over a ten year period and the culling of nearly 67,000 animals (Lyra, 2006). By the late 1990s South America and the Caribbean were declared ASF free.

On May 22, 2007 the Republic of Georgia notified the OIE of a swine disease outbreak thought to be post-weaning multisystemic wasting syndrome. On June 4th the OIE reference laboratory in Pirbright confirmed a diagnosis of ASFV. The likely source is contaminated waste brought in on ships to the Port of Poti from eastern Africa. The outbreak spread rapidly, with 56 out of 61 Georgian districts affected by July 2007 (Rowlands et al., 2008). Backyard pig farms and free range pigs with access to untreated swill and garbage likely contributed to the spread of disease and allowed for spillover into the wild boar population. Wild boar have also played a key role in the spread of ASF in Europe, though their role in ASF epidemiology in Asia remains less understood. By late 2007 the disease had entered Russia, with wild boars near Chechnya testing positive for the virus. By 2008 the disease had entered Armenia and Azerbaijan (Rowlands et al., 2008). In 2014 ASF entered the European Union and to date cases have been reported in the EU Baltic states, Romania, Bulgaria, Hungary, and Belgium. In August 2018 the first reports of ASF came from China. The exact origins are unknown, but the virus is genetically identical to the genotype II isolates circulating in Russia, The Ukraine, and the Caucasus (Zhou et al., 2018). ASF entering China was particularly concerning, because with over 50% of the global pig population China is the single largest pig producing country in the world. The US also has significant trade involvement with China and imports millions of kilograms of feed ingredients and animal products every year. As seen in Figure 1.1, ASF spread through China at an alarming rate with most provinces affected in a matter of months. The Chinese pork industry has suffered as a result, with shortages leading to significant price fluctuations. The USDA estimates that 2020 Chinese pork production will decrease 25% and imports increase 35% compared to 2019. This is also a threat to the global supply of the blood thinning medication heparin, roughly 80% of which comes from Chinese swine (Vilanova et al., 2019). Since then, ASF has been reported in Mongolia, Vietnam, Cambodia, and North Korea. On June 20, 2019, the first outbreak of ASF

in Laos was confirmed and the disease was reported in Serbia for the first time in August 2019. A pattern of rapid spread similar to what occurred in China is being seen in other Asian countries, such as in Vietnam (Figure 1.2). As the epidemic continues, movement of animals and animal products, both legally and illegally, or deliberate introduction of ASFV as an act of bioterrorism are the most likely risks to the US. USDA APHIS regulates the handling and disposal of international garbage and controls restrictions on the importation of animal products. Products, byproducts, and animal feed are allowed to be imported from ASF-endemic areas provided they are treated in a manner proven to destroy the virus (Brown & Bevins, 2018). Products coming from regions designated as low risk can be raw, provided documentation of the region and herd of origin is provided. Despite a complex regulatory system, large numbers of products are illegally smuggled into the US every year. Between 2012 and 2016 over 68,000 items were seized by US Customs and Border Protection (USCBP), but it is likely that only a small subset of contraband is detected (Brown & Bevins, 2018). USCBP also announced on March 15, 2019 that at the Newark port of entry a seizure was conducted of 50 shipping containers holding approximately 1 million pounds of pork products illegally shipped from China. In other ASFV-negative countries, such as Australia and Japan, ASFV has been identified in pork products smuggled by air passengers. Such incidents emphasize the risk of ASFV transcontinental spread and the need for the enforcement of trade and travel regulations. The US should focus resources not only on ASF mitigation strategies, but also on addressing the effects on economics and trade resulting from the global epidemic.

The virus

African swine fever virus is a large double-stranded DNA virus and the sole member of the family *Asfarviridae* (International Committee on Taxonomy of Viruses. et al., 1995). The virion is 200nm in diameter and has a 170 to 190Kb genome. The genome contains between 151 and

167 open reading frames, though about half of ASFV genes lack a known function (Alejo et al., 2018). Several concentric domains compose the mature virus, including an outer envelope, capsid, inner envelope, core shell, and nucleoid (Salas & Andrés, 2013). The outer envelope, which contains the CD2v (EP402R) transmembrane protein, is host derived and acquired during budding from the cell (S. S. Breese & Pan, 1978; Sydney S. Breese & DeBoer, 1966). CD2v is a viral homologue of the cellular CD2 protein and binds erythrocytes causing viral hemadsorption (Rodríguez et al., 1993). The capsid is the location of the highly immunogenic major capsid protein p72 (Salas & Andrés, 2013). Based on DNA sequencing of the capsid p72 gene (B646L), along with B602L, E183L (p54), and CP204L (p30), ASFV is divided into 24 genotypes (Bastos et al., 2003; Quembo et al., 2018). Based on CD2v and C-type lectin (EP153R), ASFV can also be placed in eight separate serological groups (Malogolovkin et al., 2015). While CD2v is the only viral protein decidedly located at the outer envelope, some host proteins such as CD9 and integrin beta 1 are also detectable. CD9 and integrin beta 1 are two of 21 host proteins identified in ASFV, most of which normally localize to the cell surface (Alejo et al., 2018). These virus-packaged host proteins may contribute to evasion of host immunity, but it has been observed that the outer envelope is not necessary for infection. Several proteins with roles in viral assembly and entry localize at the inner envelope including: p17 (pD117L), p54 (pE183L), p12 (pO61R), p22 (pKP177L), pH108R, pE199L and pE248R (Alejo et al., 2018). p54, a type I transmembrane protein, is crucial for the formation of the inner envelope, while p17 is required for assembly of the capsid on top of the inner envelope (J. M. Rodriguez et al., 2004; Suarez et al., 2010). The uneven distribution of proteins between the inner and outer envelops supports the notion that the inner envelope is structurally and functionally more significant than the outer envelope during viral replication. Within the inner envelope is the core shell, a thick layer comprised of several proteins originating from two polyproteins pp220 (pCP2475L) and pp62 (pCP530R) (Alejo &

Salas, 2002). The protein S273R proteolytically cleaves these two polyproteins to yield their mature products. These include the late viral proteins p150, p37, p34, p14, p5 from pp220 and p35, p15, p8 from pp62 (Alejo et al., 2018). Though viral replication primarily takes place in cytoplasmic viral factories, ASFV undergoes a crucial nuclear step as determined by the failure of enucleated cells to support virus replication (Ortin & Viñuela, 1977). At least one viral protein (p37) and viral DNA are found in the nucleus during early infection, but localize exclusively to the cytoplasm at later times (Eulálio et al., 2007). Further information about nucleus involvement during infection and ways this pathway could be manipulated to interrupt infection are unknown. The first major step of morphogenesis is the formation of the inner envelope, which is assembled from endoplasmic reticulum derived membrane fragments (Germán Andrés et al., 1998; Rouiller et al., 1998). The capsid and core shell are assembled concomitantly, with the capsid relying on the non-structural capsid assembly protein pB602L (Cobbold & Wileman, 1998; Epifano et al., 2006). Formed icosahedral particles both with and without the dense central nucleoid can be seen within viral factories, suggesting it is likely the last step of assembly (Salas & Andrés, 2013). Mature virions exit the cell by budding, though lysis of cells also leads to the release of structurally different, but still infectious, intracellular mature virions (G Andrés et al., 2001).

Host-virus interactions

Host-virus interactions are the basis for pathogenesis and host immune response, with outcomes of ASFV infection varying markedly depending on this intricate network. Studying these interactions is important to identify steps that are crucial for viral replication and potential targets for vaccines or treatments. ASFV primarily replicates in monocytes and macrophages, but receptors involved in tropism and cell entry are unknown. There are conflicting reports on the involvement of the macrophage scavenger receptor CD163 during ASFV cell entry. Sánchez-Torres et al reported that, when sorted, a $CD163^{+}$ population of monocytes demonstrated higher

permissiveness to ASFV infection than the CD163$^-$ group (Sánchez-Torres et al., 2003). Yet, a study by another group in 2014 reported that expression of exogenous CD163 did not increase infection rates, nor allow for infection in normally non-permissive cell lines (Lithgow et al., 2014). In addition, genetically edited pigs lacking CD163 show no resistance to the virus (Popescu et al., 2017). Together, these findings do not exclude the possibility that CD163 plays a role in ASFV infection, but suggests it is not imperative for infection. Beyond CD163, the existence of subpopulations of monocytes/macrophages with different expression patterns has not been evaluated as thoroughly in swine as in humans. If such subpopulations exist it is possible they may affect susceptibility to ASFV. There are likely factor(s) yet to be determined that are responsible for ASFV's tropism for porcine macrophages.

Once inside the cell, the first feat is simply surviving the harsh environment of a macrophage. ASFV utilizes a base excision repair pathway to help offset the effects of oxidative damage (García-Escudero et al., 2003). One of the main targets of immune modulation by the virus is interferon production and response. ASFV has several genes that function to inhibit interferon response, namely belonging to two multigene families, MGF360 and MGF 505/530 (Afonso et al., 2004). Permissiveness to infection in cells pretreated with IFN I supports the notion that the virus also modulates cellular responses to IFN I and prevents induction of the antiviral state (Golding et al., 2016). Inhibiting apoptosis is another common viral strategy seen during ASFV infection. The virus has several known anti-apoptotic proteins, including a bcl-2-like protein A179L, inhibitor of apoptosis protein A224L, and p53 effector protein pE153R (Afonso et al., 1996; Hurtado et al., 2004; Nogal et al., 2001). Delaying apoptosis allows for prolonged viral replication, though in later stages of infection there is a shift towards a pro-apoptotic state. ASFV also inhibits host inflammatory responses. ASFV pA238L downregulates the TNF-α and COX-2 inflammatory pathways (Granja et al., 2009). CD2v is reported to both

inhibit host immunity and enhance virulence and is also responsible for viral hemadsorption (M V Borca et al., 1998; Pérez-Núñez et al., 2015). While progress has been made in understanding host-virus interactions, many pieces of information are missing such as barriers to infection and the functions and mechanisms of action of viral proteins.

ASFV vaccines

The lack of an effective vaccine for ASF presents a significant challenge for disease control efforts. The observation that pigs surviving ASF are able to develop protective immunity gives hope that a safe and effective vaccine may be possible, but research to date has not yielded a product fit for use in production. Our incomplete understanding of factors that confer immunity to ASFV is a significant barrier to vaccine development. There is a lack of basic knowledge about the general mechanisms of virus neutralization, as well as controversy about the role of neutralizing antibodies during ASFV infection. Studies performed over the last 50 years, including a trial using modern adjuvants, have shown that traditional inactivated vaccines fail to induce protection against ASFV infection (Blome et al., 2014; Forman et al., 1982; Stone & Hess, 1967). A number of vaccine strategies have been tested including DNA vaccines and viral vectors, with mixed results. A DNA vaccine encoding p30 and p54 fused with the extracellular domain of CD2v induced an immune response, but was not protective. However, the addition of ubiquitin expressed along with the same three viral proteins did confer partial protection in the absence of specific antibodies (Argilaguet et al., 2012; Lacasta et al., 2014). Viral vector vaccines utilizing different platforms such as modified vaccinia Ankara, alphaviruses, and adenoviruses have been found to generate specific antibody and T-cell response (Jancovich et al., 2018; Lokhandwala et al., 2017; Lopera-Madrid et al., 2017). When placed into pigs though, these preparations were not found to confer protection against challenge with virulent virus (Jancovich et al., 2018; Lokhandwala et al., 2019; Netherton et al., 2019). In addition, an

accelerated clinical course sometimes observed following vaccination suggests the possibility of antibody dependent enhancement of infection (Blome et al., 2014; Escribano et al., 2013; Sunwoo et al., 2019). Currently, live attenuated vaccines (LAV) show the most promise, albeit with barriers to practical use in the field. Several studies demonstrate LAV induced protection against virulent challenge. Two deletion mutant viruses, one lacking six genes of the MGF360/505 families and the other lacking the virulence associated gene 9GL (B119L), are capable of conferring protection against challenge with parenteral virus (O'Donnell, Holinka, Gladue, et al., 2015; O'Donnell, Holinka, Krug, et al., 2015). Interestingly, an isolate harboring both of these deletions was not protective, likely due to further attenuation and decreased replication (O'Donnell et al., 2016). In contrast, vaccination with a virus lacking both the 9GL and UK (DP96R) genes led to protection against clinical disease, though replication of the parental challenge virus was not impeded (O'Donnell et al., 2017). Despite some promising results there are several hurdles preventing practical use of LAVs in the field including: prolonged shedding of the vaccine virus, risk of return to virulence, vaccine associated sequelae, mixed levels of protection against heterologous viruses, and the necessity of a companion test to differentiate vaccinated and infected animals. Recently, it was reported that an attenuated virus utilizing ASFV Georgia07 lacking the I177L gene provided complete protection from challenge with the virulent parental strain (Manuel V Borca et al., 2020). Due to the complete protection from the epidemiologically relevant Georgia07 strain, lack of vaccine associated side effects, the ASFV-G-ΔI177L strain shows encouraging results as a vaccine candidate. Further research and field testing is needed to define the target antigens and the immune mechanisms involved with protection from ASFV infection, and to balance effectiveness and safety of new vaccines.

ASFV therapeutics and antivirals

In the absence of a vaccine, alternative therapies continue to be evaluated. While more than 90 antiviral drugs are available for use today, there remain many viruses with no functional treatment (E de Clercq & E., 2016). Nucleoside antiviral drugs such as cidofovir, pyrazofurin, and ribavirin, have shown *in-vitro* inhibition of ASFV (Erik De Clercq et al., 1986; Erik De Clercq, 2002). These nucleoside analogues are used in humans against DNA viruses such as HSV-1 and poxviruses, but their effects in pigs are unknown and cost is a likely barrier for their use. Several compounds have demonstrated the ability to partially inhibit ASFV replication *in-vitro*, such as flavonoids including apigenein, genkwanin, and genistein (Arabyan et al., 2018; Hakobyan et al., 2016, 2019). These authors reported that flavonoids are able to disrupt viral DNA replication and tubulin assembly. Flavinoids are naturally occurring secondary metabolites found in many plants and plant products such as fruits, nuts, seeds, wine, and tea. Fluoroquinolones have also shown *in-vitro* antiviral activity against ASFV (Mottola et al., 2013). While the antiviral properties of genkwanin and genistein were tested on primary macrophages, most of these experiments were performed in Vero cells using the tissue culture adapted strain, and none have been tested *in-vivo.* Cost is always an important limitation of diseases of food animals, as treatment price should not exceed the value of the animal. For this reason, less expensive naturally occurring compounds have more use potential. Another consideration for practical use of therapeutics is determining the time point at which treatment will be instigated. It has been observed that viral shedding begins about the same time point as the onset of clinical signs, suggesting that successful detection and treatment of infected animals prior to contagiousness may be unlikely (Davies et al., 2017; Guinat et al., 2014). Preemptive herd treatment instituted at surrounding farms during an outbreak to provide a security zone may be

the most judicious use. ASF outbreak situations remain difficult to control with our reliance on less than ideal strategies such as stamping out, quarantine, and trade restrictions.

Transmission

Another challenge of managing the spread of ASF comes from its use of several transmission pathways. The disease can spread by direct or indirect contact between pigs, on fomites and mechanical vectors, in contaminated feed and water, and via soft ticks (Guinat et al., 2014; Plowright et al., 1969). Appearance of severe disease occurs when the virus escapes the naturally occurring sylvatic cycle between ticks and African pigs and enters a population of domestic pigs or wild boar. While infection through direct contact between warthogs and domestic pigs has not been documented, if domestic swine access an improperly disposed carcass or are fed contaminated warthog products, infection may result (Thomson et al., 1980). Warthogs may also transport infected *Ornithodoros* ticks from burrows to areas inhabited by domestic pigs, leading to exposure (Horak et al., 1983). While efficacy of experimental infection varies notably between strains and routes of exposure, once established in a population the virus is readily transmissible between domestic pigs. This spread occurs primarily through direct contact involving the oral-nasal route or wounds. Table 1.2 shows the infectivity of various doses and strains of ASFV when administered to pigs orally, nasally, or intraoropharyngeally. There is concern that a disease introduction event may lead to the establishment of an endemic cycle in the US due to the presence of both a large population of feral swine and native species of soft ticks.

Feral swine in the United States

The United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) estimates that over 6 million feral swine exist in at least 35 states. *Sus scrofa* (includes both the Eurasian wild boar and feral pigs) and *Sus scrofa domesticus* are

highly susceptible to ASF. While transmission models of ASF involving wild boar are incomplete, it is understood that they continue to play a key role in the spread and persistence of ASF in Europe. Epidemiological studies suggest that disease incidence in local wild boar populations is a significant risk factor for farms becoming infected (Halasa et al., 2019; Nurmoja et al., 2018). However, both wild boar population density and environmental conditions should be considered when evaluating virus persistence. Cold and humid environments promote virus longevity in the environment, with some suggesting that contaminated carcasses are a more significant transmission concern than live animals (EFSA, 2015). If population density is a less important transmission factor, controlling feral swine movement may be an important strategy in addition to population control. Monitoring of feral swine behavior shows these animals will seek out interactions with domestic pigs if possible, and feral pigs are also more likely than their domestic counterparts to have access to landfills or other potentially contaminated sources of food waste (Herrera-Ibatá et al., 2017; Wyckoff et al., 2009). Historically, fencing has been used successfully to control feral swine movements in national parks and some countries (such as Denmark) are currently constructing fences to control wild boar movement (Hone & Stone, 1989; McCann & Garcelon, 2008). Unfortunately, the size of the US and the scale of the feral swine problem may make this an impractical solution. An additional consideration is the impact of expansive fencing on wildlife and resistance to such a project from environmentalists. Management of wild boar populations is challenging due to their high reproductive rate and a population's tendency to disperse in response to extensive hunting efforts. A combination of tactics such as baiting, trapping, and hunting feral swine can be applied to control the population and animal movements. In June 2019 the USDA allocated $75 million to the Feral swine eradication and control pilot program (FSCP), a joint project between the USDA's Natural Resources Conservation Service and APHIS. The project will work with landowners in target

areas to remove feral swine, restore damage done by feral swine, and provide continued assistance for feral swine control to producers.

Vector-borne transmission

Ornithodoros is one of the five genera in family *Argasidae*, which is comprised of about 200 species of soft ticks (Guglielmone, 2010). ASFV is maintained in Africa in a sylvatic cycle between juvenile warthogs and soft ticks of species *O. porcinus porcinus* which inhabit burrows (Plowright et al., 1969). After 1979 the *O. moubata* complex was divided into four distinct species, including *O. porcinus* (Walton, 1979). Once a tick is infected, viral replication occurs in a number of tissues, including the midgut (Kleiboeker et al., 1998, 1999). *O. porcinus* ticks are capable of harboring10^4 to 10^6 HAD_{50}/tick, with no effect to the tick, except during the stress of oviposition (Kleiboeker et al., 1998; Plowright W., Thomson G. R. et al., 1994; Wilkinson et al., 1988). In other tick species, such as *O. coriaceus,* increased nymph mortality is observed. The two most likely routes of virus transmission to swine are through tick saliva or coxal fluid (Kleiboeker et al., 1998; Plowright et al., 1970). The United States is host to several species of *Ornithodoros* ticks including *O. coriaceus, O. parkeri, and O. turicata*. Two species, *O. coriaceus* and *O. turicata*, are able to transmit ASFV to domestic pigs (Cooley & Kohls, 1944; Groocock et al., 1980; Hess et al., 1987). *O. puertoricensis* is a species found in the Caribbean and is also a competent vector for ASFV (Endris et al., 1991, 1992). A number of soft tick species exist in the US that have not been evaluated for ASFV competence including: *Argas sanchezi, A. radiatus, A. persicus, A. giganteus, A. brevipes, A. ricei, A. miniatus, A. monolakensis, Carios capensis, C. denmarki, Otobius megnini, Otobius lagophilus, Ornithodoros kelleyi, O. hermsi, O. concanensis, O. stageri, O. talaje, O. yumatensis, O. sparnus, O. coprophilus, O. dyeri, O. rossi, O. dugesi,* and *O. quilae* (Cooley & Kohls, 1944; Donaldson et al., 2016). Based on documented geographic range and vector-host interactions

many of these ticks may be considered low risk for ASFV transmission, but they can't be completely ruled out as a transmission risk (Golnar et al., 2019). Soft ticks contribute to disease persistence by their ability to harbor and transmit ASFV for five years or more, as well as the ability of some species such as *O. porcinus* to maintain it independently of swine via transstadial and transovarian transmission (Boinas et al., 2011; Hess et al., 1989; Plowright W., Thomson G. R. et al., 1994). *O. coriaceus* is capable of transstadial transmission, but *O. parkeri* and *O. turicata* have not been evaluated for these traits. In the Iberian peninsula, the presence of the competent vector *O. erraticus* (also known as *O. marocanus*) complicated disease eradication efforts (Plowright W., Thomson G. R. et al., 1994). The presence of infected ticks necessitates drastic changes to disease control methods. For example, current EU directives increase the mandated quarantine period of a farm from 40 days after an outbreak is controlled to six years if tick vectors are present. The entry of ASFV into the soft tick population in the US holds the potential to markedly increase the difficulty of disease control.

Variable clinical disease presentation and carrier animals

African swine fever clinical presentation can be distinctly different depending on virus and host factors, with chronic or persistent disease more challenging to detect. When highly virulent strains such as Georgia07 are introduced into naïve animals, acute disease with high mortality is seen. Clinical signs of acute disease include pyrexia, crowding behavior, depression, anorexia, dyspnea, coughing, nasal discharge, erythema, cyanosis, epistaxis, vomiting, mucoid to bloody diarrhea, and abortion (Gómez-Villamandos et al., 2013; Sánchez-Vizcaíno et al., 2015). Mortality of acute disease approaches 100% within 4-8 days, with typical gross pathologic changes including ascites, hepatic and biliary congestion, hyperemic and enlarged spleen, lymphadenopathy, and petechial hemorrhage within the renal cortices, urinary bladder, epicardium, endocardium, and pleura (Gomez-Villamandos et al., 1995; Sánchez-Vizcaíno et al.,

2015). Once an outbreak is established the disease course tends to shift from acute to subacute, and over longer periods of time (as in endemic areas) chronic and subclinical disease may be observed (Allaway et al., 1995; Fasina et al., 2010; Owolodun et al., 2010). Subacute disease presents with more mild clinical signs and mortality ranges from 30 to 70% with survivors recovering in 3 to 4 weeks after infection (Sánchez-Vizcaíno et al., 2015). Chronic ASF has been primarily associated with lower virulence strains, and it has been proposed that the chronic disease seen in the Iberian peninsula from 1960 through the 1990s was a consequence of live attenuated vaccine use (Sánchez-Vizcaíno et al., 2012). Exposure to a low dose of highly virulent virus may delay the onset of disease, but mortality is not decreased. Petrov et al. reported that sentinel pigs remained ASFV negative when co-mingled with convalescent pigs at 99 days post-challenge with ASFV Netherlands86. Virus was successfully isolated from clinical samples collected from the challenged animals at 48 days post infection (dpi), and viral genome detectable in blood at 91dpi (Petrov et al., 2018). It is impossible to say if this result was due to a decline in infectiousness of the convalescent animals, or if 30 animals was not a large enough sample size to demonstrate what may be a rare transmission event. In direct contrast, Eblé et al. reported that transmission from carrier animals resulted in new acute cases when naïve pigs were co-mingled with animals recovered from ASFV Netherlands86 at 28-41dpi or 42-55dpi (Eblé et al., 2019). One consideration from these studies is that variation in virus strain will have an impact on the outcome of infection and the number of animals that survive and may become carriers. The genotype I ASFV strain Netherlands86, is considered moderately virulent, but the currently circulating genotype II strain is highly virulent. Despite this, the detection of antibody positive wild boar in Russia and eastern Europe indicates a population of animals are surviving infection (Mur et al., 2016; Nurmoja et al., 2017; Woźniakowski et al., 2016). Although the percent of positive samples are low, it indicates the possibility of feral swine acting as long term

carriers of the disease. Even in the absence of long-term carrier animals the appearance of less prominent forms of the disease, such as chronic and subacute, may play a role in persistence and spread. In addition to various forms of ASF, other diseases such as classical swine fever and erysipelas can mimic ASF. As such, a presumptive clinical diagnosis by an observant producer or veterinarian must be confirmed with both virologic and serologic diagnostic methods.

Diagnostics and surveillance

The diversity of ASF clinical presentation makes laboratory diagnosis an essential tool for disease surveillance and control. It is important to utilize both virus and antibody detection methods. For conducting diagnosis and surveillance, the preferred methods are real-time PCR for the detection of nucleic acid and ELISA for detecting antibodies.

Virologic assays

PCR is preferred because it is rapid, specific and can detect ASFV prior to the appearance of clinical signs. In surviving pigs, virus can persist for months, making PCR practical as a long-term testing strategy. The principal drawbacks of PCR include high cost and the requirement of expensive equipment for conducting high throughput screening. The OIE describes validated methods for both conventional and real-time PCR tests (Fernández-Pinero et al., 2013; Tignon et al., 2011). Currently the US has limited testing capabilities, with the USDA National Animal Health Laboratory Network (NAHLN) having 11 labs approved to test for ASF. The USDA estimates that at current capacity only 6,500 PCR samples could be run per day, with whole blood and tonsil being the only approved samples (the USDA is working to validate testing of oral fluids). For reference, the USDA quarterly estimated the pig population in the United States to be 75.5 million animals as of June 27, 2019 and a single large farm may have more than 5,000 pigs (USDA, 2019a). In the face of an epidemic where rapid largescale testing is crucial for disease control measures, our current testing capabilities would likely prove insufficient. Other

available antigen tests include direct fluorescent antibody test (FAT) and enzyme-linked immunosorbent assay (ELISA), though PCR has widely replaced these two assays and been demonstrated as more specific than antigen detecting ELISA (C. Gallardo et al., 2015). Though antigen capture ELISA is not as sensitive as PCR, it is useful for efficient screening at the herd level (C. Gallardo et al., 2019). The p72 capsid antigen is an ideal target for ASFV antigen detection because it is conserved among strains and highly immunogenic. Among the 68 proteins that comprise the virion, p72 accounts for 10% of the antigen mass (Alejo et al., 2018). While antigen tests are a useful diagnostic tool, one clear shortfall is that detecting viral DNA does not equate to infectious virus. Based on the OIE manual, virus isolation remains the preferred method for ASFV identification, but it is impractical. Propagation of ASFV requires primary swine leukocytes which are time consuming and expensive to obtain, and their use comes with ethical concerns related to animal use. In addition ASFV is a foreign animal disease that is regulated by the federal select agent program (FSAP) in the US, thus few facilities have the biocontainment requirements and expertise to propagate live virus. When virus isolation is performed, hemadsorption (HAD) reaction is commonly assessed. HAD is the term for the binding of erythrocytes and rosette formation, a phenomenon not seen with other swine pathogens and an indicator of an ASFV positive sample. It is important to note that some strains of ASFV do not cause HAD, and an additional confirmatory antigen test such as FAT should be executed on HAD negative samples. HAD testing is the OIE gold standard diagnostic, but is performed in limited amounts due to facility requirements, time required (7-10 days), need for highly trained staff, and cost.

Serologic assays

In contrast, serologic tests are inexpensive and relatively easy to perform. In the absence of available vaccines antibody presence is always indicative of exposure, though it's worth

noting that acute disease may lead to death before detectable levels of antibodies are produced. The technique of choice for antibody detection is ELISA with a follow up test such as immunoblotting (IB), indirect fluorescent antibody (IFA), or immunoperoxidase (IPT) to confirm positive samples. ELISA is a fast and low cost test, but confirmatory testing is important because incorrectly handled or stored sera can yield false positive results. IPT is more sensitive than ELISA and capable of detecting lower antibody titers, but is more labor intensive, costly, and requires use of live virus (M. C. Gallardo et al., 2015). To detect antibodies, IPT utilizes antigens produced on plates of cells infected with ASFV. The main limitation of IPT is the strict requirements required by the FSAP to remove inactivated ASFV from BSL-3 containment. This bars the notion of sending prepared and fixed plates for IPT to standard diagnostic laboratories in an efficient manner. Table 1.1 provides a brief overview of current ASF diagnostic methods. Diagnostic tools for use in the field are available, but show limited sensitivity compared to gold standard laboratory methods (C. Gallardo et al., 2019). These are useful for emergency response, but not as standalone test. Active surveillance is another tool that until recently, was missing from our diagnostic strategy. In May 2019 the USDA announced the addition of ASF to the previously existing CSF surveillance program. The USDA began CSF surveillance in March 2006 with a goal of testing targeted populations based on the following criteria: swine suspicious for CSF, sick pigs submitted to a veterinary diagnostic lab, pigs condemned at slaughter, feral swine, and swine in herds considered high risk. The selection criteria will remain the same, but the samples will now be tested for both CSF and ASF. While we have the tools to confidently diagnose ASF, we lack the ability to reach that confidence in a rapid manner and at the capacity necessary for rapid response to an outbreak. Performing multiple diagnostic tests takes time, as does the requirement to send samples to approved testing facilities. An accurate diagnosis is a multifaceted approach that should include virologic and serologic results within the context of

the clinical and epidemiological characteristics being observed in the field. Rapid response and diagnosis is key to handling an outbreak, particularly in the absence of other prophylactic measures.

Integration of existing knowledge into a biosecure US swine industry

If ASFV enters the US there are several points of concern from a biosecurity standpoint, such as limiting domestic pig and wild boar contact, controlling spread of virus by human activities, and management of environmental virus persistence. The diagram in Figure 3 illustrates the biosecurity challenges facing the US swine industry. According to a 2006 survey by the USDA APHIS, over half of farrowing, nursery, and grower/finisher sites keep pigs under total confinement, with the majority of these sites using some form of all-in/all-out management. Despite this, the US has seen recent outbreaks of swine diseases, such as the introduction and rapid spread of porcine epidemic diarrhea virus (PEDV). PEDV entered the United States in 2013 and led to the death of approximately 7 million pigs over the next year. The disease spread quickly throughout the Midwest, with epidemiological analysis suggesting that transport equipment was a major contributing risk factor (Bowman et al., 2015). Though the PEDV outbreak was primarily among domestic pigs, other outbreaks have spread via contact between feral and domestic pigs, such as brucellosis in New York in 2016. Such examples indicate that the US pig industry is still highly susceptible to outbreaks caused by new pathogens. One novel system under development is known as The Secure Pork Supply (SPS), a collaborative plan between swine producers, state and federal government officials, Iowa State University, and the University of Minnesota (www.securepork.org). The SPS is a voluntary program that ensures the implementation of disease prevention and outbreak preparedness with the goal of minimizing interruptions to business. Aspects of the SPS plan include enhanced biosecurity practices, designation of individuals who will manage surveillance and sample collection, a diagnostic

sample submission plan, and maintenance of accurate animal movement records. An example of some of the higher level biosecurity practices required by the SPS is an establishment of a perimeter buffer area and implementation of lines of separation with designated access points. The software and database for the SPS, when completed for use, will compile and share real-time industry data to provide the most up to date information on swine health status and disease outbreaks.

Conclusion

Although ASF is not present in the US, the scope of the current global outbreak has drawn considerable attention to the disease. ASF is devastating to the swine industry in affected areas, and remains difficult and expensive to control. Much is still unknown about barriers to infection, viral protein functions, and host-virus interactions. Further research is needed on these topics, with a particular focus on processes that occur early in replication, such as cell entry. The scope of this research was to evaluate virus entry pathways, compare a surrogate virus (vaccinia) to ASFV, and reevaluate the controversial role of CD163 in ASFV cell entry.

Figure 1.1 African swine fever dissemination in China

ASF spread rapidly throughout China following its introduction in August 2018. By February 13, 2019, the affected regions included: Liaoning, Henan, Jiangsu, Zhejiang, Anhui, Heilongjiang, Inner Mongolia, Jilin, Tianjin, Shanxi, Yunnan, Hunan, Guizhou, Chongqing, Jiangxi, Fujian, Hubei, Sichuan, Shangai, Beijing, Shaanxi, Qinghai, Guangdong, Guansu, Ningxia

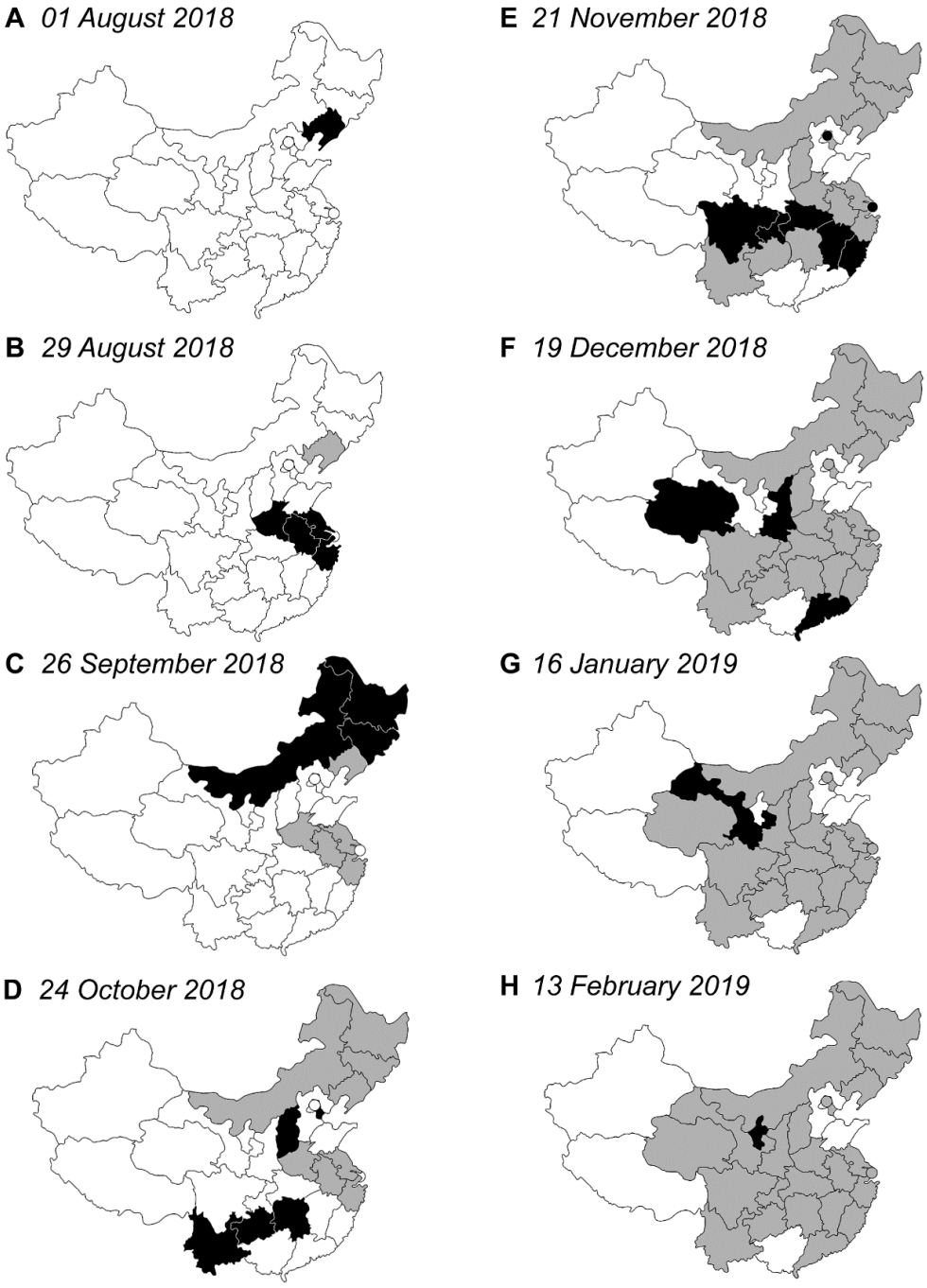


Figure 1.2 African swine fever dissemination in Vietnam

ASF spread rapidly throughout Vietnam following its introduction in February 2019 and by June the majority of the country was affected, leading to the culling of almost 3 million pigs.

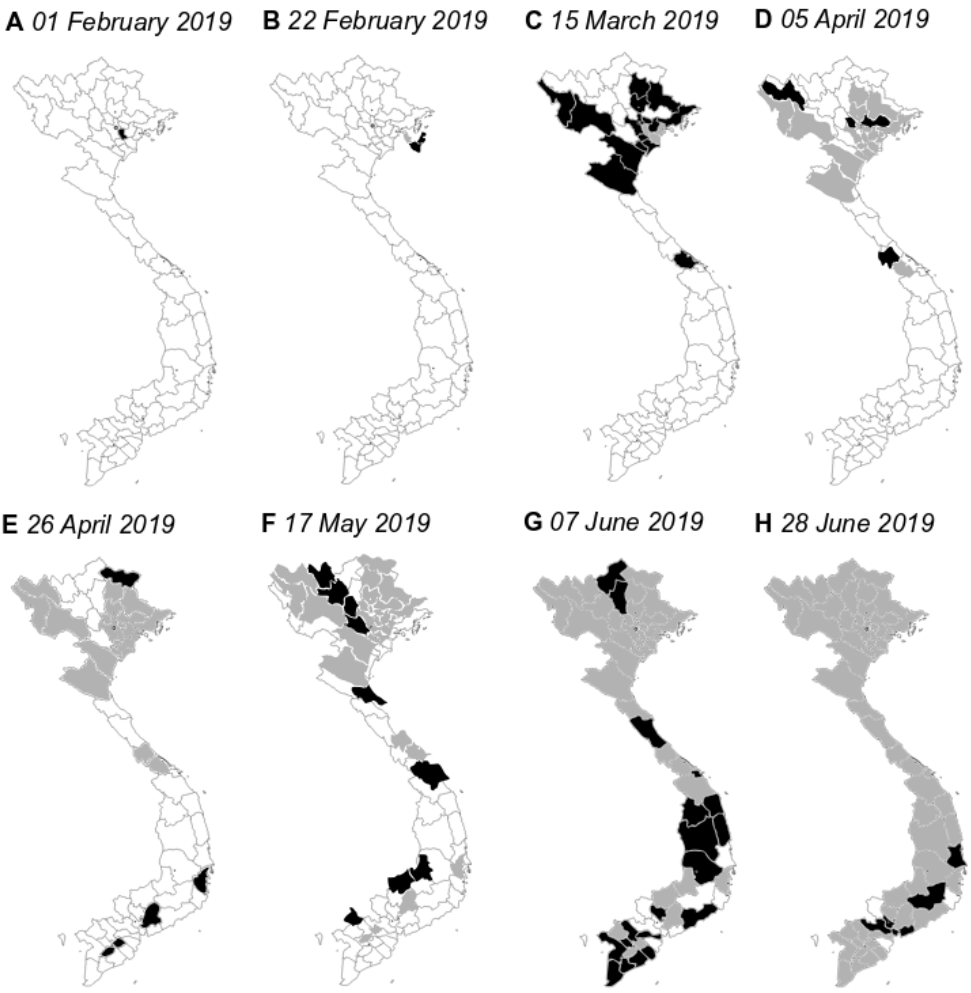


Figure 1.3 ASFV structure based on tissue culture adapted BA71V strain

The localization of 40 viral proteins among the five structural domains of the ASFV particle is shown. The distribution of proteins marked with an asterisk was inferred from the predicted or known role. Figure from (Alejo et al., 2018).

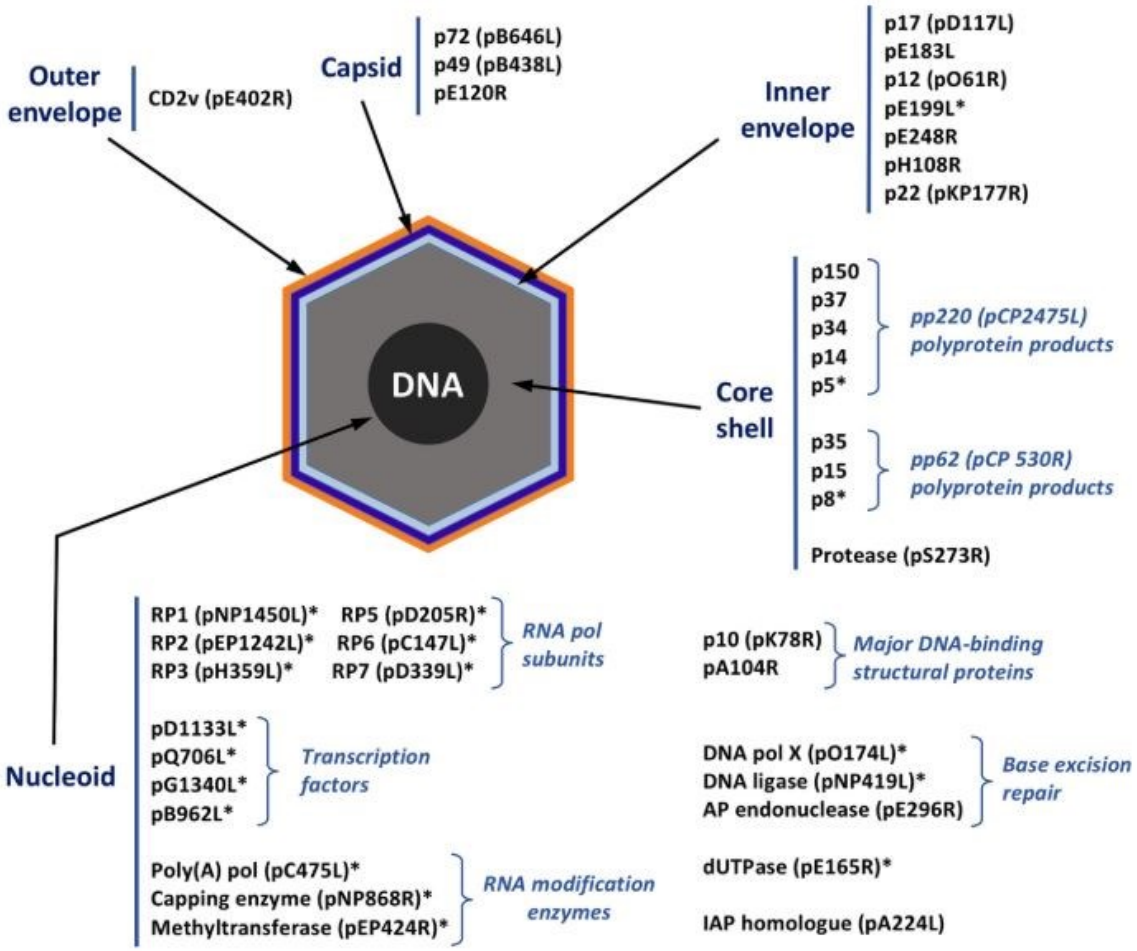


Figure 1.4 Model for ASFV internalization and uncoating

ASFV enters swine macrophages by clathrin-mediated endocytosis (left) and constitutive macropinocytosis (right). After the uptake, incoming particles are transported from early endosomes or macropinosomes to late endosomes, where they undergo a pH dependent uncoating process. Then, the exposed inner viral envelope fuses with the endosomal membrane to deliver genome-containing naked cores into the cytosol. Figure from (Hernáez et al., 2016) used under CC BY 4.0.

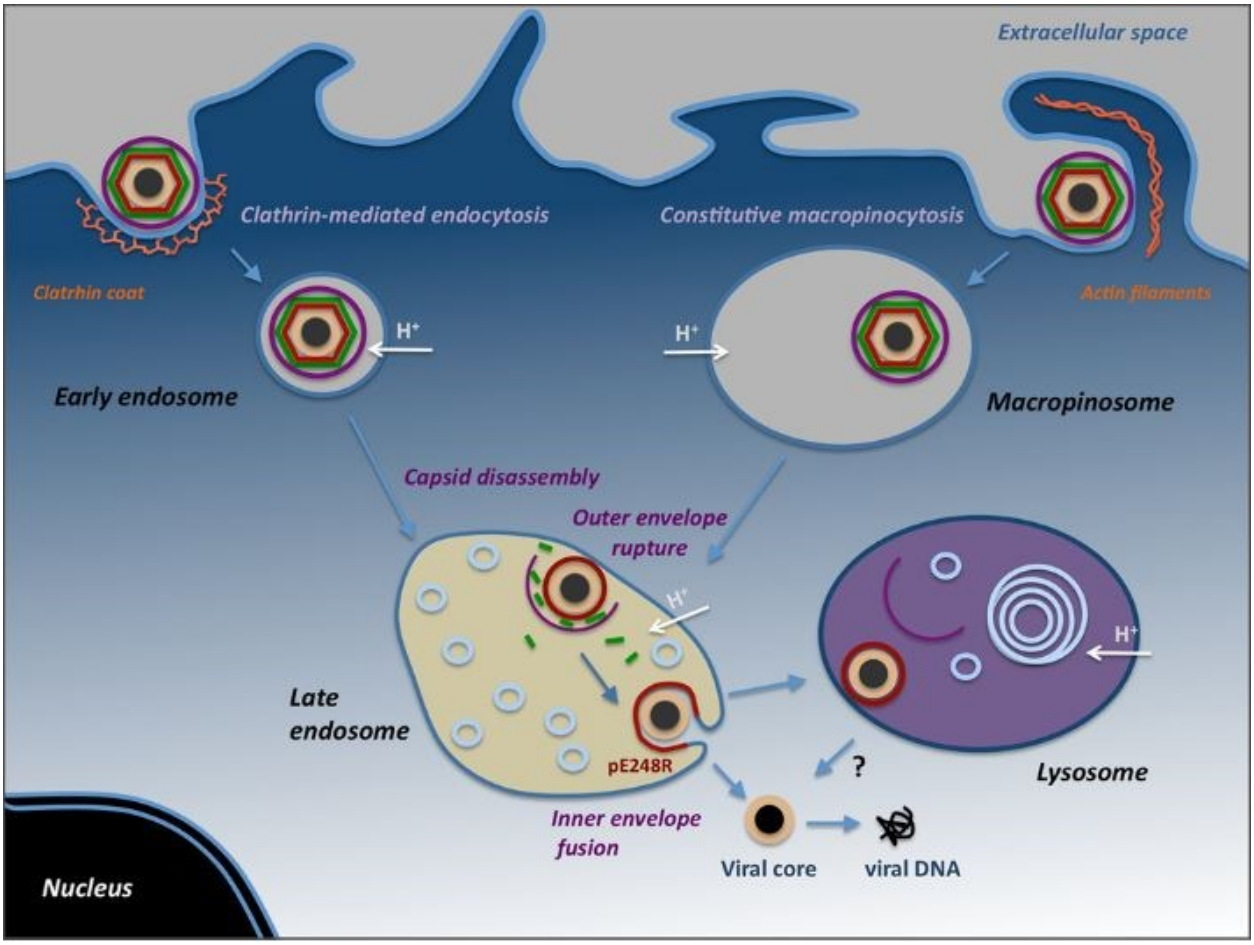


Table 1.1 African swine fever diagnostic techniques

Modified from: African swine fever: detection and diagnosis – A manual for veterinarians (Beltrán-Alcrudo et al., 2017)

Assay (Virological)	**Time**	**Cost**	**Sample required**	**Comments**
Polymerase chain reaction (PCR)	4-6 hours	Medium	Tissues, blood, ticks, cell culture supernatant	Common methodology. Nucleic acid detected may be from live or dead virus.
Hemadsorption (HAD) test	7-21 days	High	Primary porcine cell culture	**Gold standard.** Limited number of facilities can perform.
Fluorescence antibody test	1-2 hours	High	Tissues: cryosection or impression smear. Requires florescence microscope.	Reagent availability limited. Not routinely used.
ELISA	3-4 hours	Low	Serum, tissue	Not routinely used.
Assay (Serological)	**Time**	**Cost**	**Sample required**	**Comments**
ELISA	3-4 hours	Low	Serum	Recommended screening test
Immunoblotting	3-4 hours	High	Serum	Confirmatory test. No commercial kits.
Indirect fluorescent antibody test	3-4 hours	Medium	Serum, Plasma, Tissue. Requires florescence microscope	Confirmatory test. No commercial kits.

Table 1.2 Infectivity of various ASFV doses and strains when administered orally, nasally, or intraoropharyngeally

ASFV can be transmitted orally or intranasally with varying results depending on dose and strain. Using intranasal inoculation doses as low as 101 are capable of causing infection, while Malta78 achieved 100% infection at 104 and Tegani at 105. Oral inoculation seems to be less efficient, with Tanzania KWH/12 and Hinde WH II requiring a dose of 107 to achieve 100% infection.

ASFV Strain	Route	Dose	% infection	Reference
Armenia 2008	Oronasal	10^1 to 10^2	25%	Pietschmann 2015
Malawi 1983	Intraoropharyngeally	10^2	0%	Howey 2013
Malawi 1983	Intranasopharyngeally	10^2	50%	Howey 2013
Dominican Republic 1979	Intranasal/oral	$10^{2.6}$	0%	McVicar 1984
East Africa	Intranasal	$10^{2.9}$	50%	Parker 1969, reviewed in Guinat 2016
East Africa	Intranasal	$10^{2.9}$	Minimum ID	Plowright 1969
Malta'78	Intranasal	10^3	60%	de Carvalho Ferreira 2012, de Carvalho Ferreira 2013
Netherlands	Intranasal	10^3	60%	de Carvalho Ferreira 2013
Netherlands	Intranasal	$10^{3.5}$	60%	de Carvalho Ferreira 2012
Dominican Republic 1979	Intranasal/oral	$10^{3.6}$	13%	McVicar 1984
East Africa; tissues from naturally-infected warthogs	Liquid or moistened solid feed	$10^{3.7}$-$10^{6.1}$	0%	Plowright 1969
Unknown	Intranasal	$10^{3.7}$ – $10^{3.9}$	100%	Plowright 1969
Tanzania KWH/12	Spleen given orally in milk	10^4	0%	Greig 1972
Netherlands	Intranasal	10^4	60%	de Carvalho Ferreira 2013
Malta'78	Intranasal	10^4	100%	de Carvalho Ferreira 2012
Malta'78	Intranasal	10^4	100%	de Carvalho Ferreira 2013
Brazil	Intranasal	$10^{4.5}$	100%	de Carvalho Ferreira 2012
Dominican Republic 1979	Intranasal/oral	$10^{4.6}$	88%	McVicar 1984
Malawi 1983	Intranasopharyngeally	10^4 to 10^6	100%	Howey 2013
Malawi 1983	Intraoropharyngeally	10^4 to 10^6	100%	Howey 2013
Tanzania KWH/12	Spleen given orally in milk	10^5	37.5%	Greig 1972
Unknown	Oral feeding (pig tissues)	10^5	Minimum ID	Maurer 1954, reviewed in Heuschele 1967
Tengani strain	Intranasal	10^5	100%	Heuschele 1967
Dominican Republic 1979	Intranasal/oral	$10^{5.6}$	90%	McVicar 1984
Tanzania KWH/12	Spleen given orally in milk	10^6	75%	Greig 1972
Tanzania KWH/12	Spleen given orally in milk	10^7	100%	Greig 1972
Hinde WH II	Oral inoculation of infective blood	10^7-$10^{7.5}$	100%	Colgrove 1969
Hinde WH II	Minced spleen and liver added to feed	10^7-$10^{7.5}$	100%	Colgrove 1969
Tanzania KWH/12	Spleen given orally in milk	10^8	100%	Greig 1972
Kenya	Oral (Contaminated feces & urine)	Not known	100%	Montagomery 1921, reviewed in Guinat 2016
Kenya	Oral (Contaminated sweet potatoes or bananas)	Not known	0%	Montagomery 1921, reviewed in Guinat 2016
Georgia	Oral fresh grass and seeds contaminated by secretions from infectious wildboar	Not known	Epidemiological evidence; unknown dose	EC 2014, reviewed in Guinat 2016

Table 1.3 Distribution of CD163 positive macrophages in the most commonly studied human tissues

The presence of subpopulations of CD163 positive macrophages has not been studied in swine, but could make a difference in ASFV infection. Adapted from (Fabriek et al., 2005).

Tissue	Macrophage subpopulation	CD163
Spleen	Red pulp macrophages	+
	Perifollicular macrophages	-
Lymph nodes	Medullary macrophages	+
	Perifollicular macrophages	+
Thymus	Medullary macrophages	+
	Cortical macrophages	+
Liver	Kupffer cells	+
Brain	Perivascular macrophages	+
	Meningeal macrophages	+
	Microglia	-
Lung	Alveolar macrophages	+
	Interstitial macrophages	+
Blood	Monocytes	+ (10-30%)

Chapter 2 - Vaccinia virus as a model for African swine fever virus entry

Introduction

African swine fever is a unique virus and thus exists in its own family, but this was not always the case. ASFV was in family *Iridoviridae* until it was removed to an unassigned genus, *African swine fever virus group* (later renamed *African swine fever-like viruses*). In 1998 the genus was renamed to *Asfivirus* and assigned to family *Asfarviridae* (International Committee on Taxonomy of Viruses. et al., 1995; Simmonds et al., 2018). ASFV is classified as a nucleocytoplasmic large DNA virus (NCLDV), which are a group of apparently monophyletic viruses that include the following families: *Ascoviridae, Asfarviridae, Iridoviridae, Marseilleviridae, Mimiviridae, Phycodnaviridae, and Poxviridae*. ASFV has intermediate properties of both family *Poxviridae* and *Iridoviridae*. These three virus families all encode their own transcription machinery, including RNA polymerase subunits, transcription factors, and share a large number of conserved genes (Tidona & Darai, 1997; Yáñez et al., 1995).

Poxviruses are a family of complex viruses characterized by a large linear dsDNA genome, a complex enveloped structure, and cytoplasmic replication. They are some of the largest animal viruses and may be visualized using light microscopy. *Poxviridae* is split into two subfamilies, *Chordopoxvirinae* and *Entomopoxvirinae.* Sub-family *Chordopoxvirinae* contains the genus *Orthopoxvirus*, which includes vaccinia virus, variola virus, and monkeypox virus. While some poxviruses, such as variola, are host specific, others are zoonotic and can infect multiple species. Vaccinia virus (VV) is one such zoonotic member of *Orthopoxvirus,* known primarily for its role in smallpox vaccination. Vaccinia virus has been identified as a desirable surrogate for ASFV because of the similarities between ASFV and poxviruses. Vaccinia virus is

widely used and readily available, is not a select agent, and does not require high containment laboratory space.

Vaccinia virus has a 195kb genome that encodes nearly 200 proteins, and is considered the prototypical poxvirus. Despite being the most studied orthopoxvirus, the origin and natural hosts of vaccinia virus remain unknown. Some hypotheses include evolution from variola virus, evolution from cowpox virus, a hybridization of variola and cowpox, or the extinction of a species that acted as a natural vaccinia reservoir (Bedson & Dumbell, 1964; Elwood, 1989). ASFV and VV have genomic and structural similarities, with some equivalent genes involved in nucleic acid modification and replication (Yáñez et al., 1995). Both viruses are large, enveloped DNA viruses that primarily replicate in the cell cytoplasm and package many of their required enzymes. Their extensive viral transcriptional machinery provides a degree of independence from host processes and control over viral gene expression. Homology between the two viruses is referenced to support predictions of ASFV protein functions. Mature vaccinia virions have several proteins associated with the poxviral entry/fusion complex, including A16, A21, A28, F9, G3, G9, H2, J5, L1, and L5 (Sobhy, 2017a). These entry/fusion complex proteins mediate virus-cell fusion and membrane disruption. Four of these proteins share structural and sequence closeness with two ASFV transmembrane proteins. ASFV pE248R is similar to VV protein L1 and ASFV pE199L is similar to VV proteins G9, A16, and J5 (Germán Andrés, 2017; Hernáez et al., 2016). Another example of similarity is the shared ability of ASFV and VV to modulate and interfere with host immune responses, such as blocking interferon and regulating cell death. ASFV pE152R contains a complement control module/short consensus sequence domain that is also found in the VV proteins BR5 and VCP (Manuel V. Borca et al., 2016). BR5 and VCP act to inhibit complement activation (Bernet et al., 2011; Rosengard et al., 1999). Vaccinia demonstrates some of the same immune evasion tactics used by ASFV, such as regulation of

interferon, inflammation, and apoptosis (Nichols et al., 2017; Smith et al., 2018). ASFV and VV both have complex replication cycles that produce multiple forms of infectious particles. Vaccinia mature virus (MV) particles are surrounded by one membrane, while extracellular enveloped virus (EV) are surrounded by an additional membrane obtained during cell egress (Hollinshead et al., 1999). It has been proposed that this host-derived membrane may aid in evading host antibodies and complement. The majority of infectious particles are MV, which remain within the cell until lysis (Smith et al., 2018). A small portion of MV particles give rise to intracellular enveloped virions (IEV) which are triple enveloped particles wrapped by a trans-Golgi derived membrane-cisterna (Schmelz et al., 1994). EV and MV particles contain different sets of viral proteins in their outer envelopes, but due to the majority of particles being MV and the fragility of the EV outer membrane, most studies of vaccinia have focused on MV (Ichihashi, 1996).

Knowledge of specific host-cell receptors that interact with poxviruses is limited, though some relevant cellular proteins have been evaluated. Studies from multiple groups have reported on the role of cellular glycosaminoglycans during MV attachment and entry. Chung et al. first reported the ability of vaccinia to interact with heparin sulfate (HS), with binding occurring between HS and VV proteins A27 and H3 (Chung et al., 1998; Lin et al., 2000). Chondroitin sulfate, another glycosaminoglycan, is reported to interact with the viral protein D8 and HeLa cells lacking glycosaminoglycans are less susceptible to VV infection (Chung et al., 1998; Hsiao et al., 1999; Lin et al., 2000). The lipid raft associated protein CD98 has also been found to play a role in viral entry, with infection rate being reduced 50% in HeLa cells with CD98 knockdown (Sobhy, 2017b). CD98 is an amino acid transport and cell adhesion glycoprotein expressed by most cell types (Verrey et al., 2000). Some genes have been identified as poxviral host-range

genes, with the E3L and K3L genes of vaccinia being the best characterized (Seet et al., 2003). These proteins both play a role in immune evasion.

Unlike many other viruses, poxvirus tropism at the cellular level seems to be regulated by downstream intracellular events, instead of by receptors at the level of binding and entry. Viral entry into both permissive and restrictive cells has been observed, but replication is abortive in the latter (Johnston et al., 2003). An example of this can be seen using K3L deletion mutant VV, which undergoes abortive infection specifically in BHK-21 cells (Beattie et al., 1995; Langland & Jacobs, 2002). As such, poxviruses can enter a wide variety of mammalian cells, but permissiveness to replication varies between cell lines and virus strain. General mechanisms of viral entry include virus-cell fusion, cell-cell fusion, and endocytosis. There are several endocytic pathways, including phagocytosis, pinocytosis, and receptor-dependent pathways. Vaccinia has primarily been reported to enter cells via macropinocytosis, however there are contrasting reports surrounding VV entry mechanisms, which may be explained by variations between both the virus strain and cell type used. HeLa, BHK-21, and BSC-1, are three commonly used cell lines with VV. Vaccinia virus entry into HeLa and BSC40 (a variant cell line derived from BSC-1) cells has been described as using a dynamin-dependent endocytic pathway, or dynamin-independent macropinocytosis (Huang et al., 2008; Mercer & Helenius, 2008b).

The poxvirus intracellular replication cycle has been most studied on vaccinia, but essential features are highly conserved within the virus family. Once inside the cell, both ASFV and VV rely on microtubules for intracellular transport to the site of uncoating and formation of viral factories (Carter, 2003; Hernaez et al., 2006). Though vaccinia virus interactions with Vero cells are less studied, differences from other cell lines have been reported. Viral kinetics are cell type dependent, with Vero cell entry occurring faster than other cell lines. Whitbeck et al.

reported that entry into Vero cells was detectable by 3 minutes, versus 6 to 9 minutes for HeLa and BSC-1 cells. Similar to ASFV, vaccinia can utilize a low pH dependent endosomal entry pathway with infectivity enhanced in BSC-1 cells at a pH between 4 and 4.5 (Townsley et al., 2006). However, compared to HeLa and BSC-1 cells, vaccinia entry into Vero cells is less sensitive to inhibition using bafilomycin, an inhibitor of endosomal acidification (Whitbeck et al., 2009). This suggests that in some cells VV may utilize both pH-dependent and pH-independent pathways. Such alternate pathways may be responsible for the faster rate of entry into Vero cells. We hypothesized that vaccinia will exhibit similar cell entry characteristics into Vero cells as BA71V, the Vero adapted strain of ASFV.

Materials and Methods

Cells and viruses.

Vero (ATCC CCL-81) cells were purchased from the American Type Culture Collection (ATCC). BHK-21 cells (ATCC CCL-10) and a green fluorescent protein (GFP) tagged vaccinia virus were kindly provided by Zhilong Yang of the Division of Biology at Kansas State University. DH82 (ATCC CRL-10389) were kindly provided by Roman Ganta of the Division of Diagnostic Medicine and Pathobiology at Kansas State University. The Vero adapted strain of African swine fever virus BA71V was previously grown in our lab. All cell cultures were maintained in a humid incubator at 37°C containing 5% CO_2. DH82 cells and BHK-21 cells were maintained in minimum essential medium with Earle's salts and L-glutamine (Corning #10-010-CM) and supplemented with penicillin/streptomycin (Gibco #15070063) at 160 U/mL, amphotericin B (Gibco #15290026) at 3 ug/mL, and 10% fetal bovine serum. Vero cells were maintained in minimum essential medium with Earle's salts and L-glutamine and supplemented with penicillin/streptomycin (160 U/mL), amphotericin B (3 ug/mL), and 7% fetal bovine serum. Vaccinia virus stocks were produced using BHK-21 cells. After 48-72 hours, infected cells were

detached using a cell scraper and pelleted using low speed centrifugation, lysed by rapid freezing and thawing following resuspension of the pellet, vortexed, and aliquoted and stored at -80C° as previously described (Reeder et al., 2016). Prior to use, each batch of harvested virus was tested via endpoint dilution to determine the $TCID_{50}$/mL as calculated using the Spearman-Karber method (Hierholzer & Killington, 1996).

Cell inhibitor compounds

Seven inhibitory compounds were used in this study (Table 2.1). All compounds were reconstituted in DMSO and stored in aliquots at -20°C. EIPA (Tocris Bioscience CAS#1154252) was stored as a 5 mM solution. Chlorpromazine (Sigma-Aldrich CAS#69090) was stored as a 50 mM solution. Cytochalasin D (Sigma-Aldrich CAS #22144770), ML-7 (Sigma Aldrich CAS#110448334), and Wortmannin (Cayman Chemical CAS#19545267) were stored as 1 mM solutions. Nocodazole (Cayman Chemical CAS#31430189) and Dynasore (Cayman Chemical CAS#304448553) were stored as 5 mM solutions.

Cell Viability

Viability of chemically treated cells was determined using MTT [3-(4,5-dimethylthiazol-2- yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay. MTT (Cayman Chemical CAS#57360697) was reconstituted in sterile PBS to a stock concentration of 5 mg/mL and stored in single use aliquots at -20°C. A 96 well plate of cells was treated with a range of concentrations of each chemical inhibitor and incubated at 37°C and 5% CO_2 for 6 hours. Following incubation the media containing drug was removed from all wells. Cells were washed once with sterile PBS and then 100 uL of fresh media containing 0.5 mg/mL of MTT reagent was added. Cells were incubated at 37°C and 5% CO_2 for 3-4 hours until purple formazan crystals visualized. The MTT reagent containing media was removed and 100 uL of 100% DMSO added to dissolve the crystals. After 10 to 15 minutes of incubation the optical density

was recorded at 570nm using a microplate reader. Reported results are from two experiments with each sample tested in duplicates.

Fluorescently labeled dextran uptake

Fluorescently labeled dextran (ThermoFisher, Alex Fluor 488, 10000MW, anionic, fixable) was reconstituted using sterile PBS to a 2 mg/mL solution and stored in aliquots at -20°C. DH82 cells were treated with concentrations of dextran ranging from 6.25 ug/mL to 200 ug/mL and incubated for 24 hours. The cells were observed under a fluorescence microscope at various timepoints to visualize particle internalization. All incubation was done in an incubator at 37°C and 5% CO_2. EIPA was used to examine the ability to inhibit macropinocytosis and the fluid phase uptake of dextran particles. DH82 cells or Vero cells were treated with concentrations of EIPA ranging from 25uM to 1000uM. After a 30 minute incubation 200 ug/mL of dextran was added. After 30 a further minutes of incubation the cells were washed with PBS and fixed for 10 minutes using 4% paraformaldehyde. The number of fluorescent particles was counted in the cells observed in two microscope fields and represented as the average number of particles per cell.

Vaccinia virus infection inhibition

24 well plates of 70-80% confluent cells were treated with the each inhibitor across a range of concentrations (see Table 2.1). Following treatment, cells were incubated for 30 minutes. Cells were then infected with 10^5 TCID50 of VV or ASFV BA71V. Treated and infected cells were incubated for 6 hours at 37C and 5% CO_2. The media was then removed. Cells were washed once with sterile PBS, and fresh media was added. Following a 24 hour incubation at 37C and 5% CO_2 cells were fixed, stained, and counted under a fluorescence microscope. Vero cells infected with BA71V were fixed with 4% paraformaldehyde for 10 minutes at room temperature followed by a 7 minute permeabilization at room temperature with

0.2% saponin. Cells were stained using a monoclonal p30 antibody at 1:6000 dilution for 1 hour at 37°C or overnight at 4°C (Petrovan et al., 2019). Goat anti-mouse IgG Alexa Fluor 488 was used as the secondary antibody at 1:400 dilution for 1 hour at 37°C. DAPI was used as a counter stain prior to viewing with a fluorescence microscope. BHK-21 cells and Vero cells infected with VV-GFP were fixed with 4% paraformaldehyde and permeabilized with 0.2% saponin. Fixed cells were incubated with PureLink RNase A at 100ug/mL for 20 minutes at room temperature, followed by counterstaining with Propidium Iodide at 1:3000 dilution for five minutes. Following staining cells were viewed with a fluorescence microscope. The number of cells and the number of infected cells were determined from an average of three randomly selected fields of view from each well. Reported results are from two experiments with each sample tested in duplicates. Error bars represent standard error between experiments. Statistical significance was calculated using the student's t-test (* = $p \leq .05$, ** = $p \leq .01$, *** = $p \leq .001$).

Results

Cell viability

The cytotoxicity of the seven selected compounds was evaluated based on MTT assay after cells were treated for six hours. This time interval was chosen because six hours was the viral absorption period used in virus inhibition experiments. As seen in Figures 2.1 and 2.2 some of the lower concentration treatments show a viability of over 100% as compared to untreated controls. This is a fairly common result with the MTT assay due to random experimental fluctuation and possible stimulation by the treatment. Hormesis is a common toxicologic phenomenon that describes a biphasic dose response characterized by stimulation at low doses and inhibition or toxicity at high doses (Calabrese & Baldwin, 2002). This observed low dose stimulation may be due to a direct effect of the compound or due to a stress related compensatory response (Calabrese et al., 2007). A compensatory response to a stressor may increase cellular

metabolism of MTT. Unlike hormesis, direct chemical reduction of MTT by tested compounds often leads to excessively high viability values (>500%). Figure 2.3 shows the MTT results for the selected concentrations that are represented on the virus inhibition figures.

Fluorescently labeled dextran uptake

Dextran was used as a surrogate particle in early experiments in order to observe macropinocytosis occurring in cells. Uptake of fluorescent dextran occurred rapidly, with intracellular particles visible as early as 5 minutes after the addition of dextran to cells. While both cell lines underwent fluid-phase uptake of dextran particles, based on the number of particles the endocytic activity appears higher in DH-82 cells than Vero cells. Both cell lines demonstrate a dose dependent decrease in particle uptake when treated with EIPA, though the inhibitory effect appears to be more potent in DH-82 cells.

Vaccinia virus inhibition in BHK-21 cells

Treatment with nocodazole and EIPA showed a significant decrease in infection.

Vaccinia virus and ASFV BA71V inhibition in Vero cells

Chlorpromazine

Chlorpromazine (CPZ) is commonly used in cell culture as an inhibitor of clathrin-mediated endocytosis. It is a cationic amphipathic drug believed to inhibit formation of the clathrin coated-pit by interfering with binding between clathrin it's associated adapter proteins (Wang et al., 1993). In Vero cells, treatment with CPZ up to 15uM did not significantly alter infection with BA71V. VV infection was reduced when cells were treated with 1.875uM of CPZ.

EIPA

5-(N-ethhyl-n-isopropil)- amiloride (EIPA) is an inhibitor of the Na+/H+ exchanger and commonly used as an inhibitor of macropinocytosis because it exerts minimal effect on other

endocytic processes. Vero cells treated with EIPA showed a decrease in infection rate of both VV and BA71V, consistent with macropinocytosis involvement in cell entry.

Cytochalasin D

Cytochalasin D (Cyto D) is a cell permeable fungal toxin that binds to the barbed ends of actin filaments. It disrupts actin filaments, and inhibits actin polymerization (Miranda et al., 1974). Cyto D disrupts the entry of both viruses, as it affects macropinocytosis due to the role actin plays in the formation and trafficking of macropinosomes.

Wortmannin

Wortmannin is a PI3 kinase inhibitor reported to reduce fluid-phase uptake via macropinocytosis (Araki et al., 1996). Despite the percent decrease of both BA71V and VV infected cells as compared to the control, due to variance between the samples the p-value was not significant.

Dynasore

The large GTPase dynamin is essential for clathrin-dependent vesicle formation during clathrin-mediate endocytosis (Kirchhausen et al., 2008). However, dynasore has also been described as having additional effects, such as reducing labile cholesterol in the cell membrane and disrupting lipid raft organization (Preta et al., 2015). Infection of both viruses was reduced in Vero cells treated with dynasore.

ML-7

ML-7 is a selective myosin light chain kinase (MLCK) inhibitor (Gao, Li-Hong Ye, Hiroko Kishi, Tsuy, 2001). MLCK phosphorylates the regulatory light chain of myosin II. Non-muscle myosin II plays a role in a number of cell processes including clathrin-mediated endocytosis and has recently been described as a potential therapeutic target during infection by

pathogenic organisms (Chandrasekar et al., 2014; Tan et al., 2019). Treatment of Vero cells with ML-7 reduced infection with both ASFV and BA71V.

Nocodazole

Nocodazole is a drug that depolymerizes microtubules and can disrupt early endosomal movement. Treatment of Vero cells with nocodazole led to inhibition of ASFV infection, but not VV.

Discussion

BHK-21 cells were used to propagate Vaccinia virus and were also tested with the inhibitors. The effect of dynasore is ambiguous with some studies reporting it to influence cell entry (Huang et al., 2008) and others finding it does not (Mercer & Helenius, 2008a). Despite a mean reduction in infection of 30%, the decrease was not found to be significant ($p = 0.54$). As expected, the Na^+/H^+ antiporter inhibitor EIPA produced the most pronounced effect on infection.

While BHK-21 is a cell line that is fairly commonly used with Vaccinia virus, Vero cells have been less studied with this virus. The inhibitor results suggest that VV is capable of utilizing both clathrin-dependent endocytosis and macropinocytosis when entering Vero cells. Though most studies have reported macropinocytosis as the primary entry pathway for Vaccinia virus, there are known variances. For example, infection of CHO cells is not affected by macropinocytosis inhibitors such as EIPA and wortmannin, suggesting alternative entry pathways (Mercer et al., 2010).

Failure of chlorpromazine to reduce BA71V infection in Vero cells, as well as the inhibitory effect of EIPA and wortmannin, suggests that macropinocytosis is the primary entry pathway for BA71V into Vero cells. Prior reports have identified chlorpromazine inhibition of clathrin as not interfering with ASFV entry, but affecting late viral protein synthesis. In contrast,

dynasore, another inhibitor that effects endosomal fusion and receptor-mediated endocytosis, does reduce ASFV infection in Vero cells. One possible explanation is due to additional potential effects of dynasore (such as affecting plasma membrane cholesterol) being responsible for the virus interference. It is also possible that clathrin-mediated endocytosis is occurring, and usage of a higher dose of CPZ would have produced a more profound inhibitory effect. Using a higher dose would likely necessitate a shorter treatment period to limit cytotoxicity. The reduced infection of VV seen following treatment of Vero cells with chlorpromazine or dynasore suggests a higher sensitivity to disruption of clathrin- and dynamin-dependent endocytosis than BA71V. It has also been reported that in HeLa cells, Vaccinia recruits clathrin to stimulate actin-motility for virus transport following fusion, which could play a role in its inhibitory effect (Humphries et al., 2012). The novel use of ML-7 with VV and ASFV also led to decreased infection rates with both viruses. This indicates that myosin II activity is involved in early infection of Vero cells with VV and ASFV BA71V.

While there are some apparent differences in interactions with the host cell during early infection, overall the effects induced by the tested inhibitors was fairly consistent between the two viruses. Future studies can appropriately use VV as a model for ASFV, but should consider the possible variance in the cellular processes utilized during early infection. Further evaluation of VV entry pathways into Vero cells or macrophages should evaluate later steps (such as the role of endosomal acidification and late viral protein synthesis) and would do well to incorporate additional testing methodologies, such as flow cytometry. Vaccinia virus infection in primary macrophages may present a better model for ASFV which still alleviates the need for BSL-3 space, but does introduce the drawback of using primary cells. Vaccinia virus will replicate in swine macrophages and Hernáez et al reported that VV infection on macrophages was unaffected by treatment with CPZ, but information beyond that are limited (Hernáez et al., 2016).

Describing effective surrogate models for ASFV is useful due to the barriers for working with virulent ASFV. Virulent strains of ASFV require primary macrophages which are time consuming to collect and have a finite lifespan in culture. Use of the virus also requires BSL-3 containment in most regions and is strictly regulated. Vaccinia virus as a surrogate virus allows for work to proceed in BSL-2, which requires less cost, time, and regulation. Knowledge of viral and cellular factors involved in the stages of early infection can help to develop new antiviral strategies.

Figure 2.1 BHK-21 cell viability based on MTT

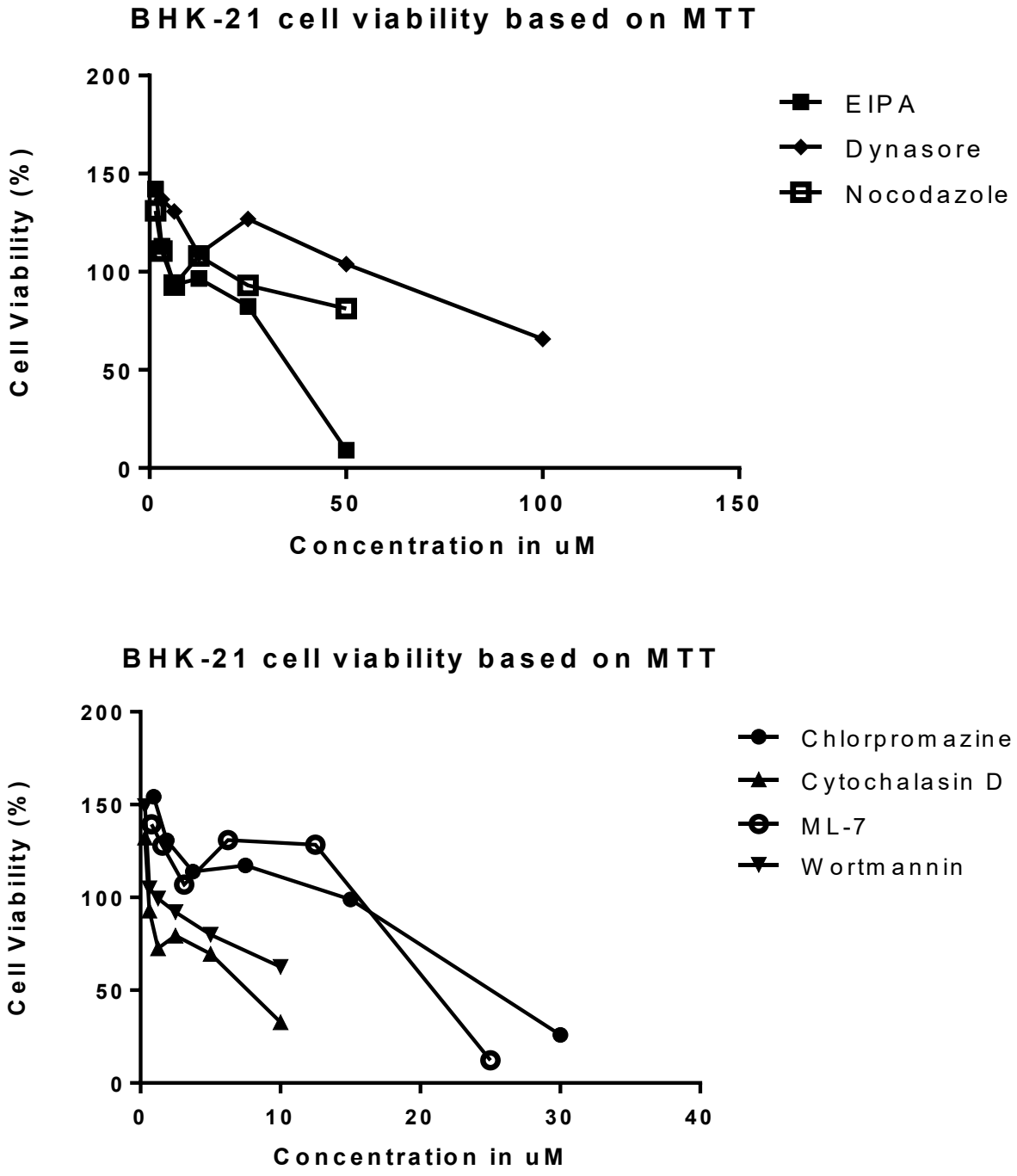


Figure 2.2 Vero cell viability based on MTT

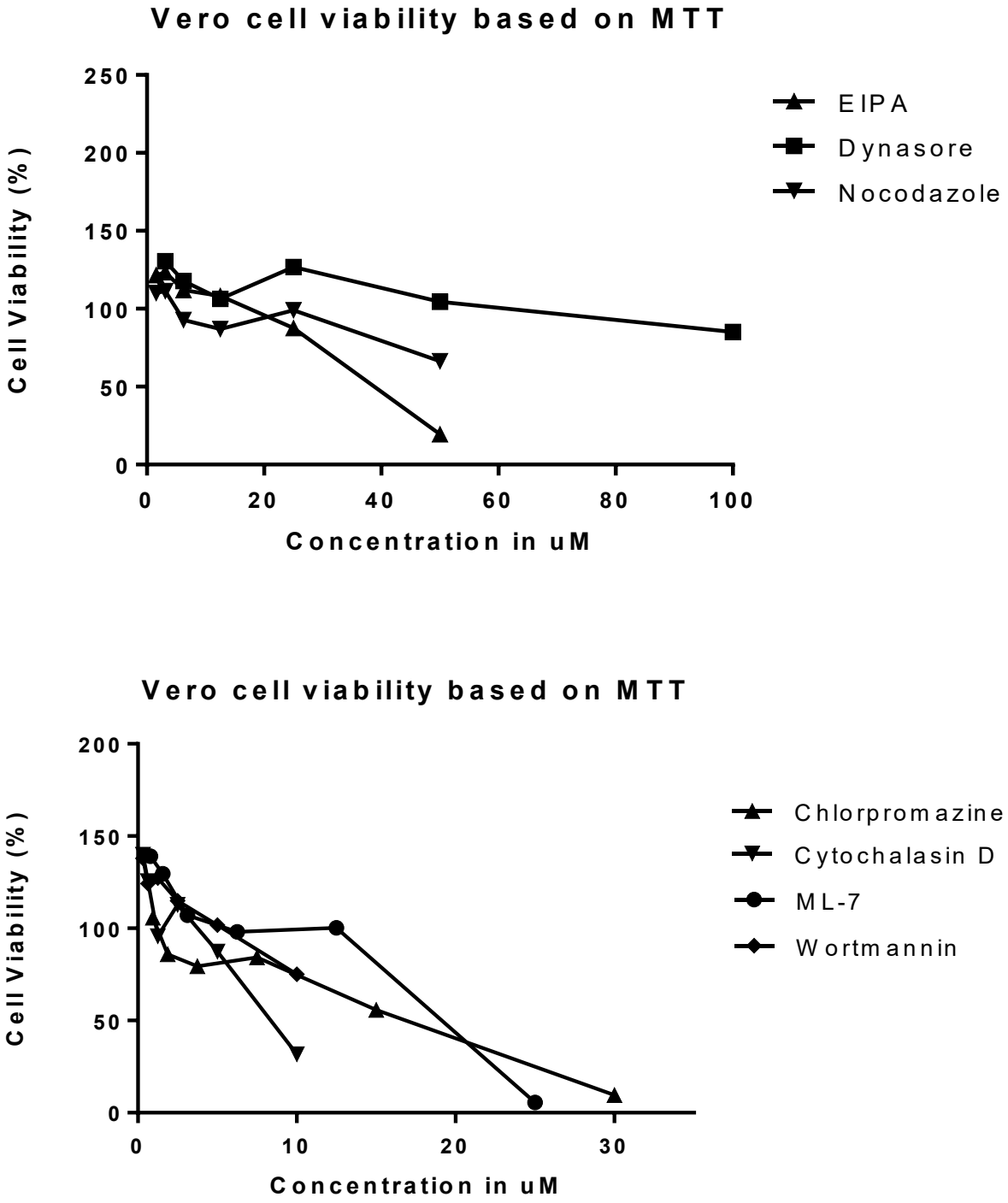


Figure 2.3 BHK-21 cell treatments with >93% viability on MTT

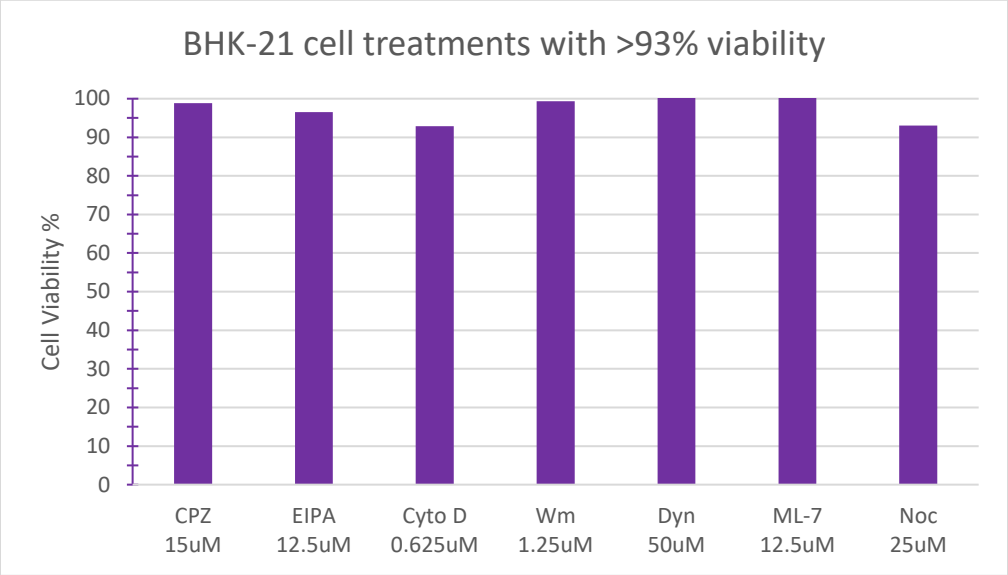


Figure 2.4 Vero cell treatments with >95% viability on MTT

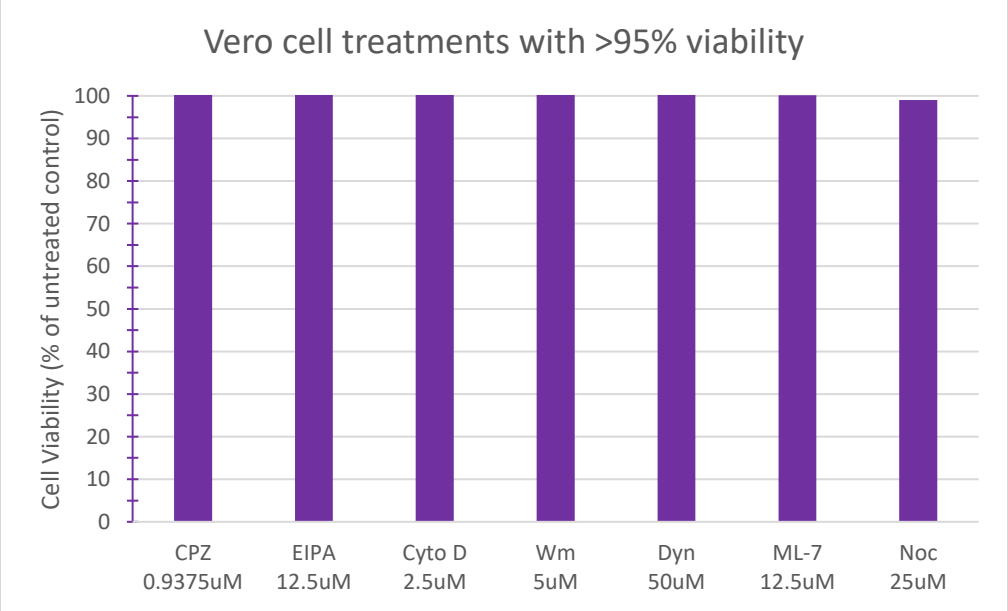


Figure 2.5 Cellular uptake and inhibition of fluorescent dextran on DH82 cells

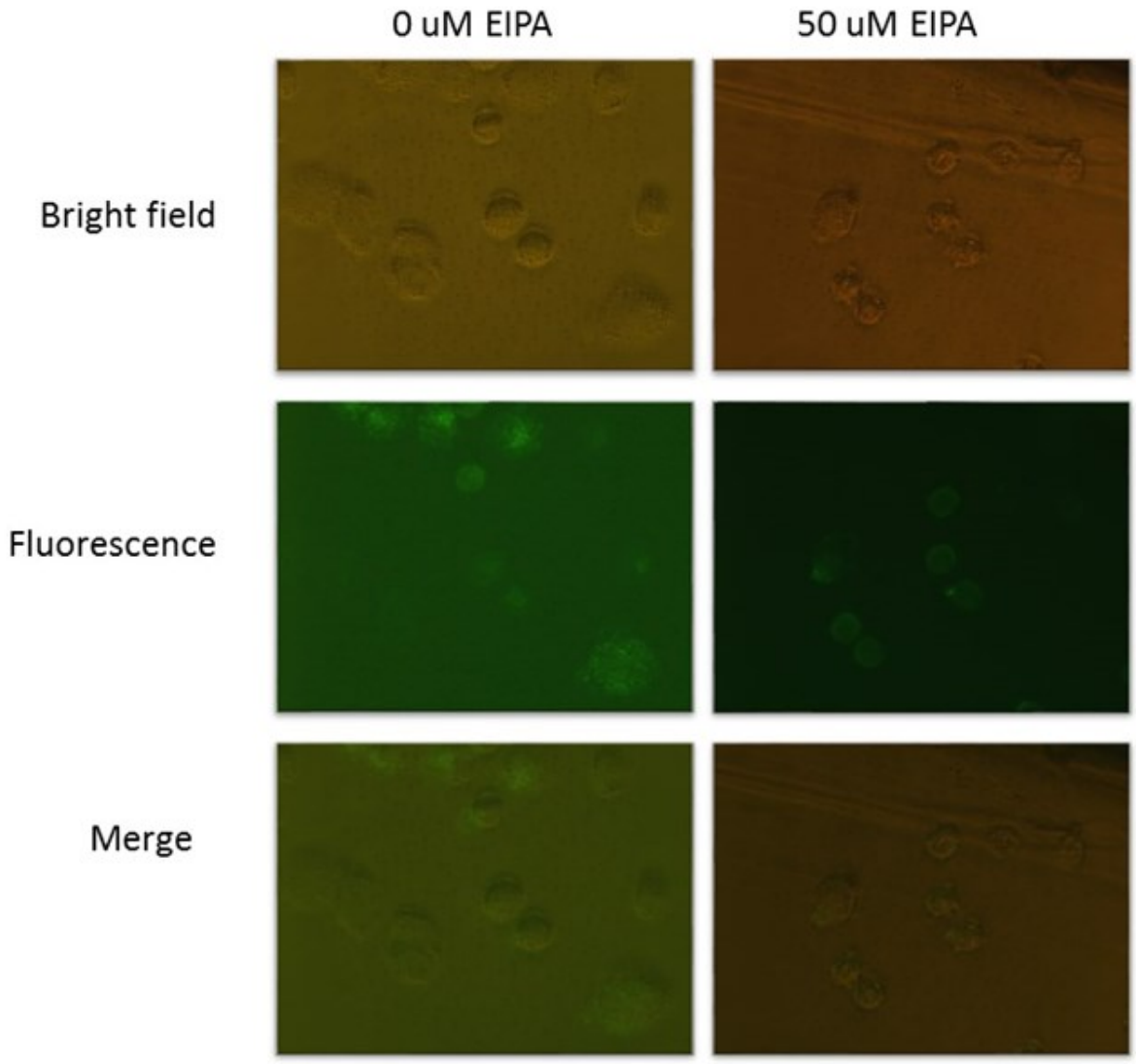


Figure 2.6 Inhibition of dextran uptake in DH82 cells

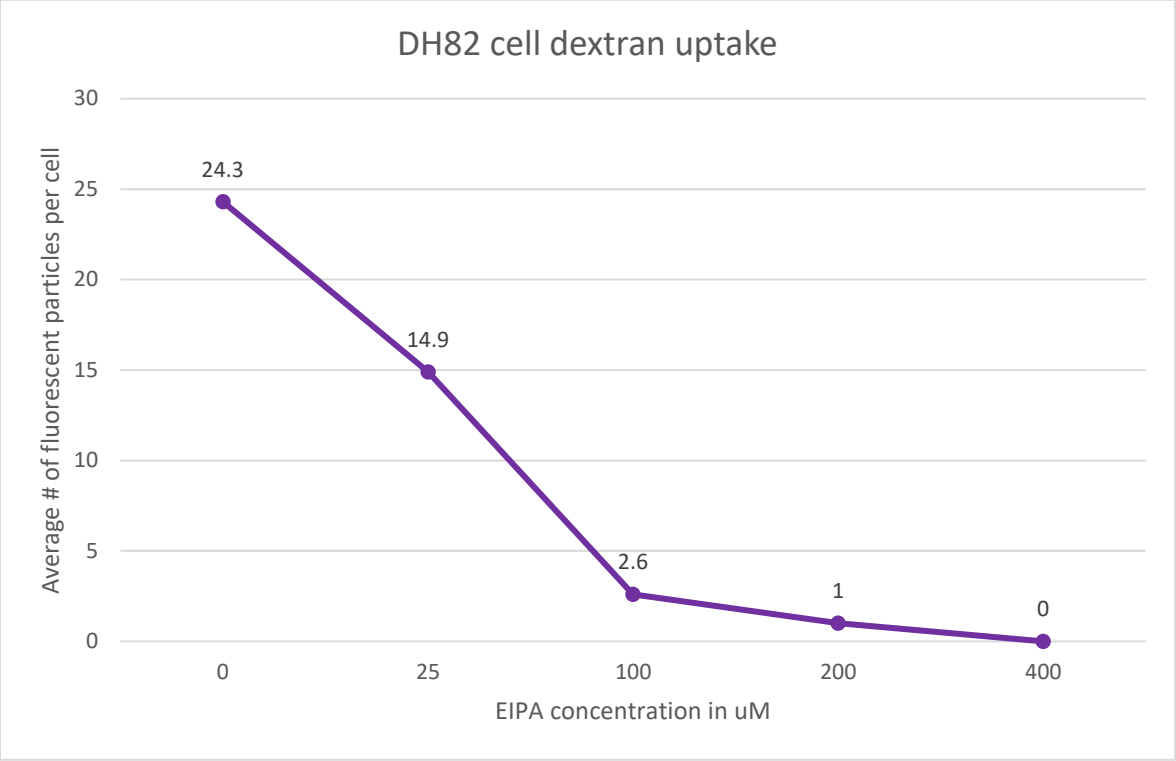


Figure 2.7 Inhibition of dextran uptake in Vero cells

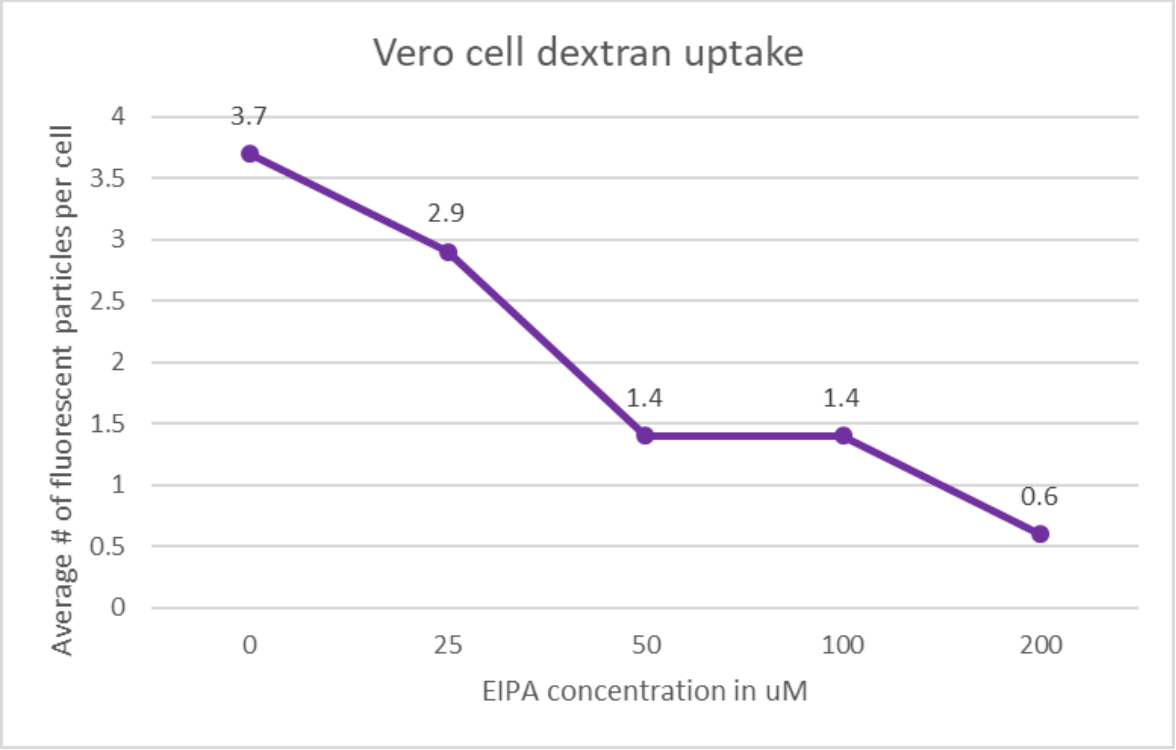


Figure 2.8 Vaccinia virus inhibition on BHK-21 cells

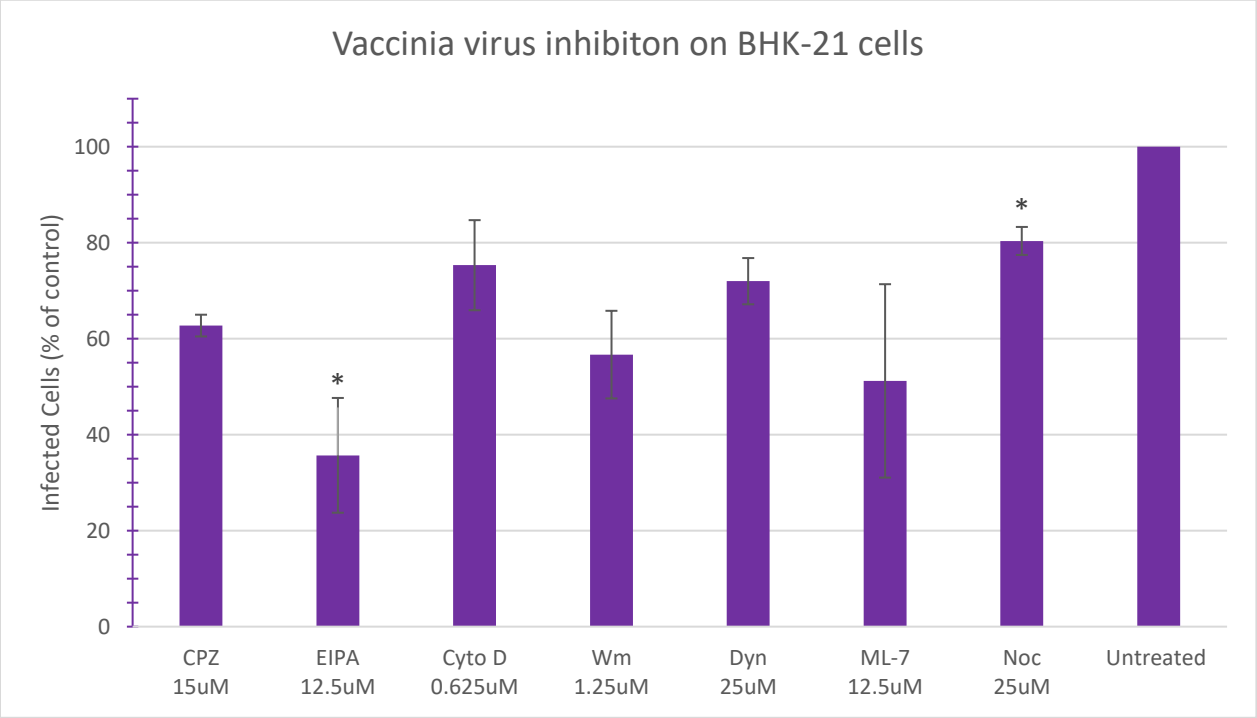


Figure 2.9 Vaccinia virus inhibition on Vero cells

(* = p ≤ .05, ** = p ≤ .01, *** = p ≤ .001)

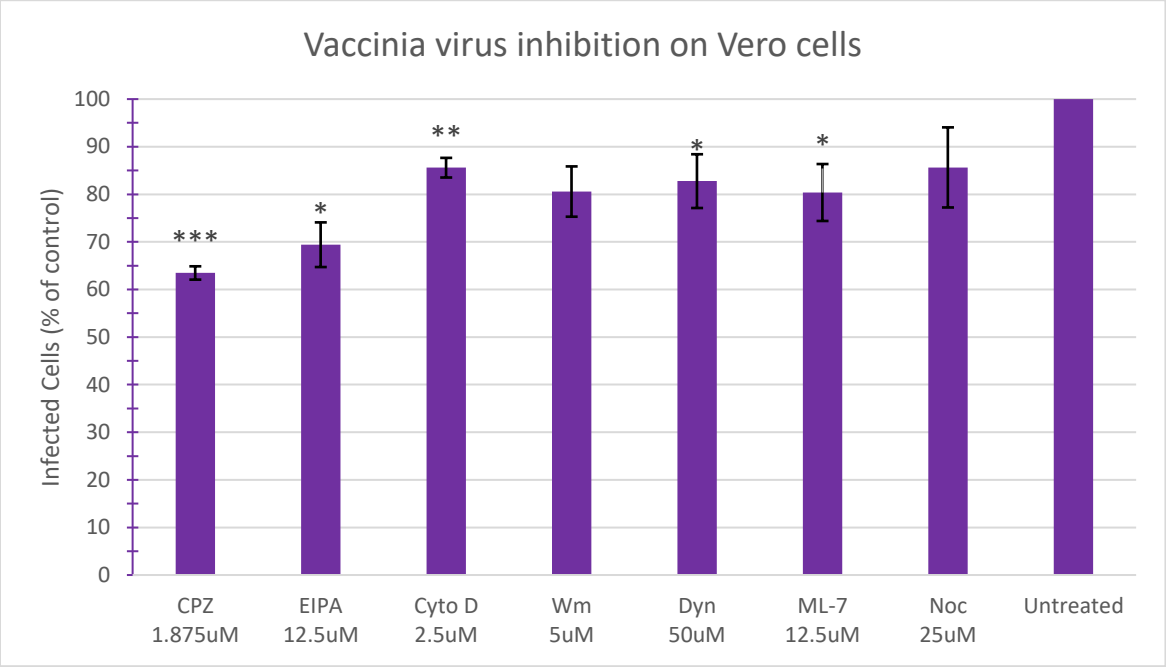


Figure 2.10 ASFV BA71V inhibition on Vero cell

(* = p ≤ .05, ** = p ≤ .01, *** = p ≤ .001)

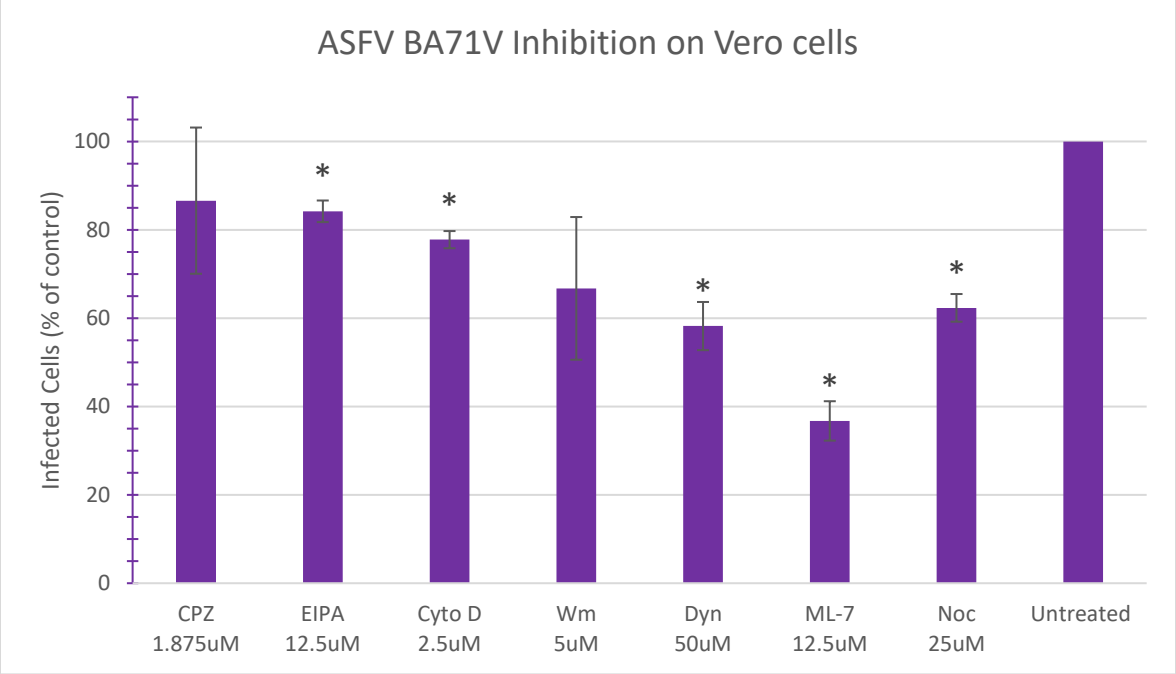


Figure 2.11 Fluorescence microscopy of Vero and BHK-21 cells infected with Vaccinia virus

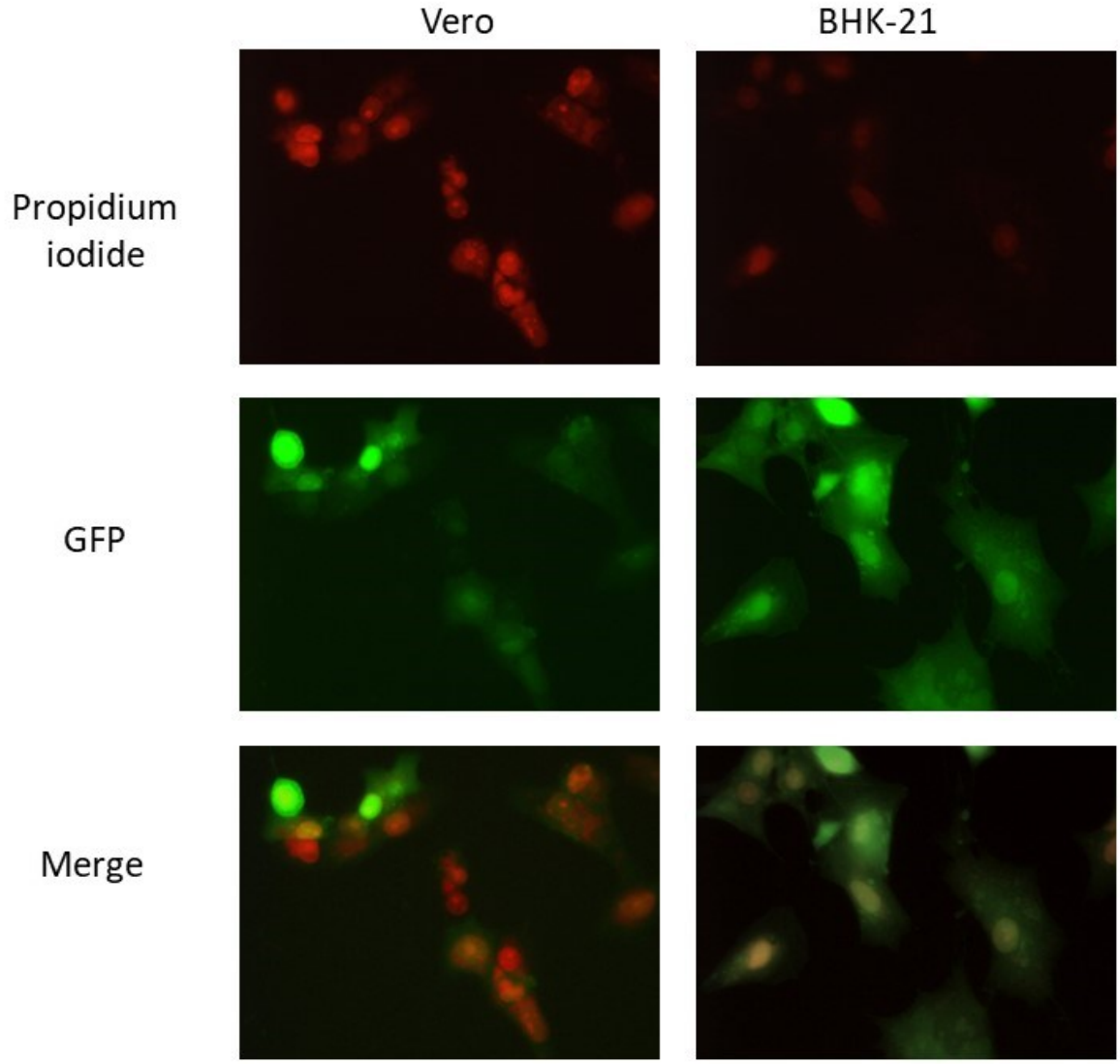


Table 2.1 Inhibitor information

Compound	Target(s)	Range of concentrations used in uM	
		Maximum	Minimum
EIPA	Na+/H+ antiport	50	1.5625
Cytochalasin D	Actin polymerization	10	0.3125
Wortmannin	Phosphatidylinositol-3 kinase	10	0.3125
Chlorpromazine	Adaptor protein-2	30	0.9375
Dynosore	dynamin1, 2, DRP1	100	3.125
ML-7	Myosin	25	0.78125
Nocodazole	Microtubule formation	50	1.5625

Chapter 3 - ASFV cell entry: macrophages and CD163

Introduction

African swine fever virus is a large double-stranded DNA virus and the only member of the family *Asfarviridae* (International Committee on Taxonomy of Viruses. et al., 1995). With a diameter of approximately 200nm it is a structurally large icosahedral shaped virus. The ASFV genome is between 170 and 190Kb and encodes for over 150 proteins. The virus consists of an outer envelope, capsid, inner envelope, and core shell surrounding the nucleoid (Salas & Andrés, 2013). Monocytes and macrophages are the main cellular targets of ASFV, however the specific cellular receptor(s) involved in viral entry and tissue tropism are unknown (Oura et al., 1998; F. Rodriguez et al., 1996). Macrophages from other species, such as humans, rabbits, guinea pigs, hamsters, and rats show no effect from the virus (Enjuanes et al., 1977). Macrophages play an important role in normal homeostasis and have activity during pathologic conditions such as infection and inflammation. Mature tissue macrophages are capable of phagocytizing and destroying foreign materials, and producing inflammatory mediators. The infectious cycle begins with viral attachment and entry into the host cell. Early studies comparing virulent and tissue culture adapted strains found that the entry mechanism is a low pH- and temperature-dependent process, which is consistent with specific receptor-mediated endocytosis (A Alcamí et al., 1990; Antonio Alcamí et al., 1989). However, receptor mediated-endocytosis appears to be necessary but not sufficient for viral infection (Carrascosa et al., 1999). CD163 is one receptor that has been purported to play a role in ASFV cell entry, but researchers have come to varying conclusions. CD163 is a scavenger receptor cysteine-rich domain (SRCR). SRCR domains are a subset of scavenger receptors, which are cell surface glycoproteins. CD163 is highly expressed on macrophages and is a known receptor for other pathogens, namely PRRVS. The best

characterized biological function of CD163 is the binding and clearance of hemoglobin-haptoglobin complexes. Free hemoglobin is oxidative, so this clearance process not only protects against damage, but may also provide a pathway for iron uptake and recycling in macrophages.

A handful of studies have evaluated the role of CD163 during ASFV infection. Sánchez-Torres et al first reported in 2003 that CD163 expression correlated with infection, and that the anti-pig CD163 monoclonal antibody 2A10 is able to inhibit ASFV binding and infection of PAMs (Sánchez-Torres et al., 2003). Yet later studies have found contradictory results, such as the inability of stable CD163 expression to allow infection in non-permissive cells (Lithgow et al., 2014). Genetically modified pigs with a complete knockout of CD163 also show no resistance to disease when challenged with virulent ASFV (Popescu et al., 2017). In addition, the ability of ASFV to replicate in cells that do not express CD163 during late infection also suggests alternative receptors or entry pathways. Together, this data shows that CD163 is not required for ASFV infection, but does not rule out its involvement. Despite a lack of consensus on which cellular factors are involved, a receptor-dependent endocytic process is believed to play a role in ASFV cell entry. It is now known that another entry mechanism used by ASFV is macropinocytosis. When treated with the macropinocytosis inhibitor, Ethylisopropyl amiloride (EIPA), cells show a dose-dependent reduction in ASFV uptake (Sánchez et al., 2012). Macropinocytosis is a non-specific cellular process for the uptake of various molecules, in which they are engulfed and endocytosed into vacuoles termed macropinosomes. While a variety of cell types can utilize macropinocytosis it is usually transient in response to growth factors, except for immune cells such as dendritic cells and macrophages which can constitutively undergo macropinocytosis (Racoosin & Swanson, 1992; Sallusto, 1995). Some viruses that utilize macropinocytosis are known to induce the process, but current evidence is more suggestive that ASFV utilizes constitutive cellular pathways (Hernáez et al., 2016; Lim & Gleeson, 2011).

ASFV's use of multiple entry pathways may be what allows the virus to infect alternate cell types during late stages of infection, such as hepatocytes, epithelial cells, and endothelial cells (Germán Andrés, 2017; Valli, 2007). Another alternative pathway that has been proposed is antibody-dependent enhancement, where antibodies facilitate cell entry via Fc or complement receptors. Despite ASFV utilizing non-specific mechanisms of cell entry, it is alleged that other factors contribute to its host and cellular specificity, such as an undiscovered protein interaction between the host and virus. We hypothesize that though it is not required, CD163 does play a role in ASFV cell entry, and that treatment with cellular inhibitors (particularly inhibitors that affect macropinocytosis) will reflect a difference in virus infection rates between wildtype and CD163 knockout macrophages.

Materials and Methods

Cells and viruses.

Pulmonary alveolar macrophages (PAMs) were collected via lung lavage. Three approximately four week old pigs were humanely euthanized by IV injection of pentobarbital. The pluck was carefully removed and the trachea incised distal to the tongue. The excised lungs were filled with 150mL to 300mL of cold sterile PBS poured from 50mL conical tubes. Following massage of the lungs, the liquid is poured back out into the sterile tubes and placed on ice until processing. Cells are pelleted via centrifugation at 500xg for 5 minutes, then washed once with sterile PBS and pelleted again. Pelleted cells were resuspended in freezing media (RPMI + 50% FBS + 10% DMSO), frozen at -80°C, then moved to liquid nitrogen for long term storage. CD163KO PAMs were harvested in the same manner from CD163KO pigs that were generated using *in-vitro* fertilization of oocytes edited by CRISPR-Cas9. See Whitworth et al 2014 for more information (Whitworth et al., 2014). Cell cultures were maintained in a humid incubator at 37°C containing 5% CO_2. PAMs were maintained in RPMI with L-glutamine

(Corning #10-040-CM) and supplemented with penicillin/streptomycin (Gibco #15070063) at 72 U/mL, amphotericin B (Gibco # 15290026) at 3 ug/mL, and 10% fetal bovine serum. Spleen homogenate from a pig infected with African swine fever Gerogia07 during a prior animal study was used as the source of virus.

Cell inhibitor compounds

Seven inhibitory compounds were used in this study (Table 2.1). All compounds were reconstituted in DMSO and stored in aliquots at -20°C. EIPA (Tocris Bioscience CAS#1154252) was stored as a 5 mM solution. Chlorpromazine (Sigma-Aldrich CAS#69090) was stored as a 50 mM solution. Cytochalasin D (Sigma-Aldrich CAS #22144770), ML-7 (Sigma Aldrich CAS#110448334), and Wortmannin (Cayman Chemical CAS#19545267) were stored as 1 mM solutions. Nocodazole (Cayman Chemical CAS#31430189) and Dynasore (Cayman Chemical CAS#304448553) were stored as 5 mM solutions.

Cell Viability

Viability of chemically treated cells was determined using MTT [3-(4,5-dimethylthiazol-2- yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay. MTT (Cayman Chemical CAS#57360697) was reconstituted in sterile PBS to a stock concentration of 5 mg/mL and stored in single use aliquots at -20°C. A 96 well plate of cells was treated with increasing concentrations of each chemical inhibitor and incubated at 37°C and 5% CO_2 for 6 hours or 24 hours. Following incubation the media containing drug was removed from all wells and replaced with 100 uL of fresh and untreated media containing 0.5 mg/mL of MTT reagent. Cells were then incubated at 37°C and 5% CO_2 for 3-4 hours until purple formazan crystals were visualized. The MTT reagent containing media was removed and 100 uL of 100% DMSO added to dissolute the crystals. After 10 to 15 minutes of incubation the optical density was recorded at 570nm

using a microplate reader. Reported results are from two experiments with each sample tested in duplicates.

Inhibition of ASFV infection on PAMs

96 well plates of PAMs were treated with the each inhibitor across a range of concentrations (see Table 2.1). Following treatment, cells were incubated for 30 minutes. Cells were then infected with a 1:100 dilution of ASFV Georgia07 spleen homogenate. Treated and infected cells were incubated for 6 hours at 37C and 5% CO_2. The media was then removed and replaced with fresh media. Following a 48 hour incubation at 37C and 5% CO_2 cells were fixed, stained, and counted under a fluorescence microscope. PAMs infected with Georgia07 were fixed with 4% paraformaldehyde for 10 minutes at room temperature followed by a 7 minute permeabilization at room temperature with 0.2% saponin. Cells were stained using a monoclonal p30 antibody at 1:6000 dilution or anti-CD163 (Mouse anti-pig 2A10) antibody at 1:100 dilution for 1 hour at 37°C or overnight at 4°C (Petrovan et al., 2019). Goat anti-mouse IgG Alexa Fluor 488 was used as the secondary antibody and incubated at 1:400 dilution for 1 hour at 37°C. DAPI was used as a counter stain prior to viewing with a fluorescence microscope. An automated program was used to image three randomly selected fields per well. The number of cells and the number of infected cells were determined from the images. The total number of cells in each image was counted using the analyze particles macro in ImageJ, while the number of infected cells was manually counted. Error bars represent standard error between experiments. Statistical significance was calculated using the student's t-test (* = $p \leq .05$, ** = $p \leq .01$, *** = $p \leq .001$).

Results

Cell viability

The cytotoxicity of the seven selected compounds was evaluated based on MTT assay after cells were treated for six hours. This time interval was chosen because six hours was the viral absorption period used in virus inhibition experiments. As seen in Figures 3.1 and 3.2 some of the lower concentration treatments show a viability of over 100% as compared to untreated controls. This is a fairly common result with the MTT assay due to random experimental fluctuation and possible stimulation by the treatment. Hormesis is a common toxicologic phenomenon that describes a biphasic dose response characterized by stimulation at low doses and inhibition or toxicity at high doses (Calabrese & Baldwin, 2002). This observed low dose stimulation may be due to a direct effect of the compound or due to a stress related compensatory response (Calabrese et al., 2007). A compensatory response to a stressor may increase cellular metabolism of MTT. Unlike hormesis, direct chemical reduction of MTT by tested compounds often leads to excessively high viability values (>500%).

Inhibition of ASFV infection on PAMs

Chlorpromazine

Chlorpromazine (CPZ) is commonly used in cell culture as an inhibitor of clathrin-mediated endocytosis. It is a cationic amphipathic drug believed to inhibit formation of the clathrin coated-pit by interfering with binding between clathrin it's associated adapter proteins (Wang et al., 1993). Treatment of PAMs with 15uM of CPZ reduced the rate of infection of ASFV in both WT and CD163KO cells. However, there was no statistically significant difference between the relative percent infection of the two cell types.

EIPA

5-(N-ethhyl-n-isopropil)- amiloride (EIPA) is an inhibitor of the Na+/H+ exchanger and commonly used as an inhibitor of macropinocytosis because it exerts minimal effect on other endocytic processes. Treatment of PAMs with 25uM or 50uM of EIPA reduced the rate of infection of ASFV in both WT and CD163KO cells. However, there was no statistically significant difference between the relative percent infection of the two cell types.

Cytochalasin D

Cytochalasin D (Cyto D) is a cell permeable fungal toxin that binds to the barbed ends of actin filaments. disrupts actin filaments, and inhibits actin polymerization (Miranda et al., 1974). Cyto D disrupts the entry of both viruses, as it affects macropinocytosis due to the role actin plays in the formation and trafficking of macropinosomes. Treatment of PAMs with 10uM of Cyto D reduced the rate of infection of ASFV in both WT and CD163KO cells. However, there was no statistically significant difference between the relative percent infection of the two cell types.

Wortmannin

Wortmannin is a PI3 kinase inhibitor reported to reduce fluid-phase uptake via macropinocytosis (Araki et al., 1996). Treatment of PAMs with 10uM of wortmannin reduced the rate of infection of ASFV in both WT and CD163KO cells. However, there was no statistically significant difference between the relative percent infection of the two cell types.

Dynasore

The large GTPase dynamin is essential for clathrin-dependent vesicle formation during clathrin-mediate endocytosis (Kirchhausen et al., 2008). However, dynasore has also been described as having additional effects, such as reducing labile cholesterol in the cell membrane and disrupting lipid raft organization (Preta et al., 2015). Infection of both cell types was reduced

following treatment with dynasore, but there was not a significant difference between the knockout and wildtype cells.

ML-7

ML-7 is a selective myosin light chain kinase (MLCK) inhibitor (Gao, Li-Hong Ye, Hiroko Kishi, Tsuy, 2001). MLCK phosphorylates the regulatory light chain of myosin II. Non-muscle myosin II plays a role in a number of cell processes including clathrin-mediated endocytosis and has recently been described as a potential therapeutic target during infection by pathogenic organisms (Chandrasekar et al., 2014; Tan et al., 2019). Treatment of both WT and CD163KO PAMs with ML-7 reduced infection with ASFV Georgia07, but with no significant difference in the relative infection rates of the two groups.

Nocodazole

Nocodazole is a drug that depolymerizes microtubules and can disrupt early endosomal movement. Treatment of cells with nocodazole led to inhibition of ASFV infection, but there was no significant difference between wildtype and CD163KO macrophages.

Discussion

Although at this time the evidence is fairly substantial that CD163 is not required for ASFV infection, there remains uncertainty if it is has any involvement with infection. While ASFV infection has been observed in other cell types, particularly during late stages of disease, the mechanism behind the viral tropism for swine macrophages remains unknown. The question remains if there is a specific cellular receptor that is crucial to cellular permissiveness to ASFV, or as if theorized with Vaccinia, tropism is primarily defined by post-entry events. We aimed to examine if there is a difference between wildtype and CD163KO PAMs in the virus-host interactions that occur during cell entry and early infection. The inhibitors used do not appear to affect cell viability differently between WT and CD163KO PAMs. While the viability curve

generated by MTT results does have some variation, at the concentrations reported for the inhibition experiments there is no statistically significant difference between the two cell types. Overall, macrophages were tolerant to higher concentrations of treatments than other cell lines tested.

ASFV enters macrophages using macropinocytosis and dynamin- and clathrin- dependent endocytosis. As expected, all of the inhibitors inhibited ASFV in macrophages, including the MLCK inhibitor ML-7. Despite the observed lower relative percentage of infection in the CD163KO PAMs, none of the differences were calculated to be statistically significant. Ultimately this aligns with the current paradigm of early infection, and suggests that CD163 is not involved at all with ASFV cell entry and early infection. While this study did not examine the inhibitor affects for the duration of infection, with CD163 being a cell surface protein it seems less likely to play a role beyond early infection. Future studies should focus on other cellular receptors that may be involved in ASFV entry, and cellular factors required for permissiveness to infection.

Figure 3.1 PAMs viability based on MTT

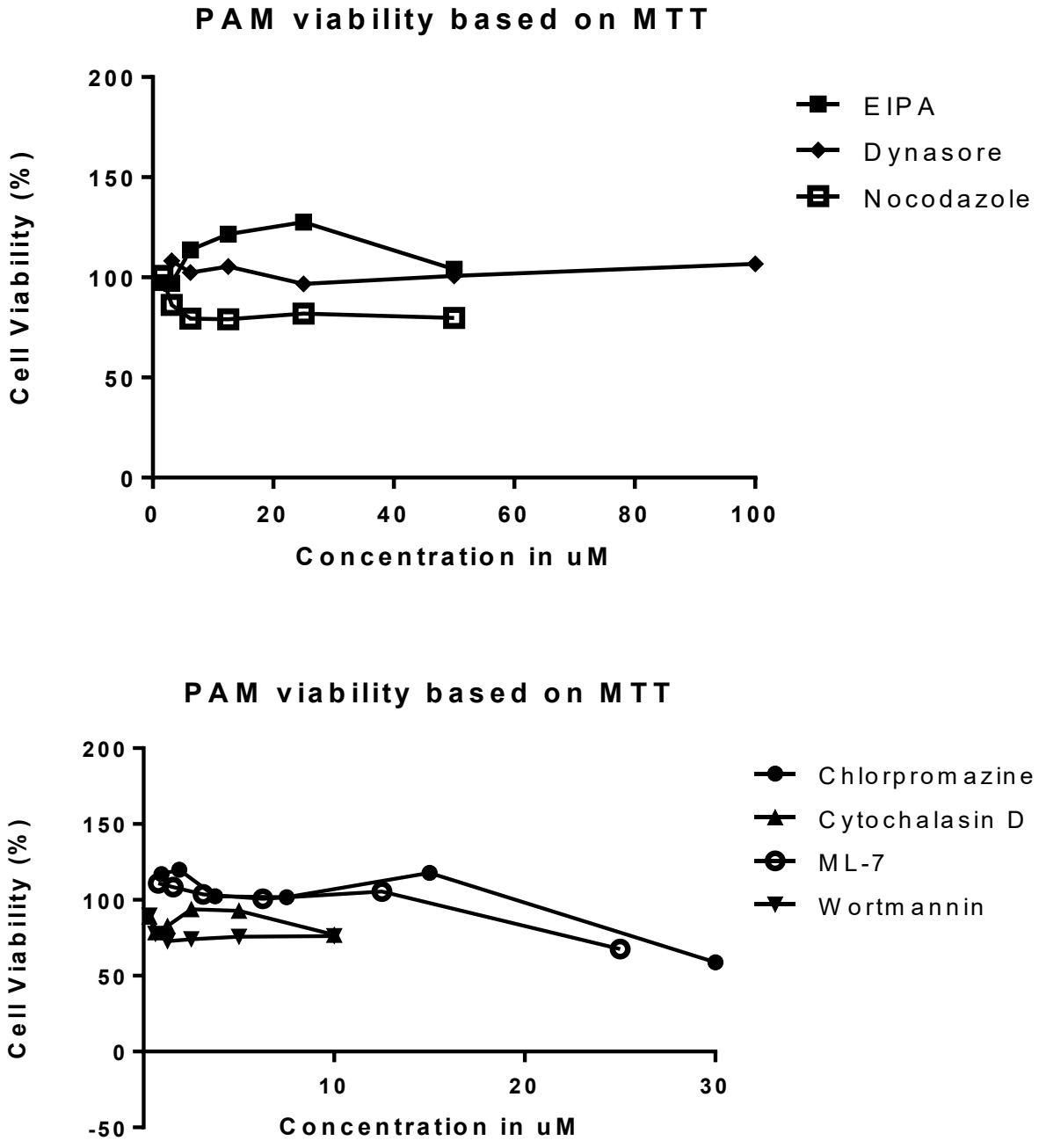


Figure 3.2 CD163 Knockout PAMs viability based on MTT

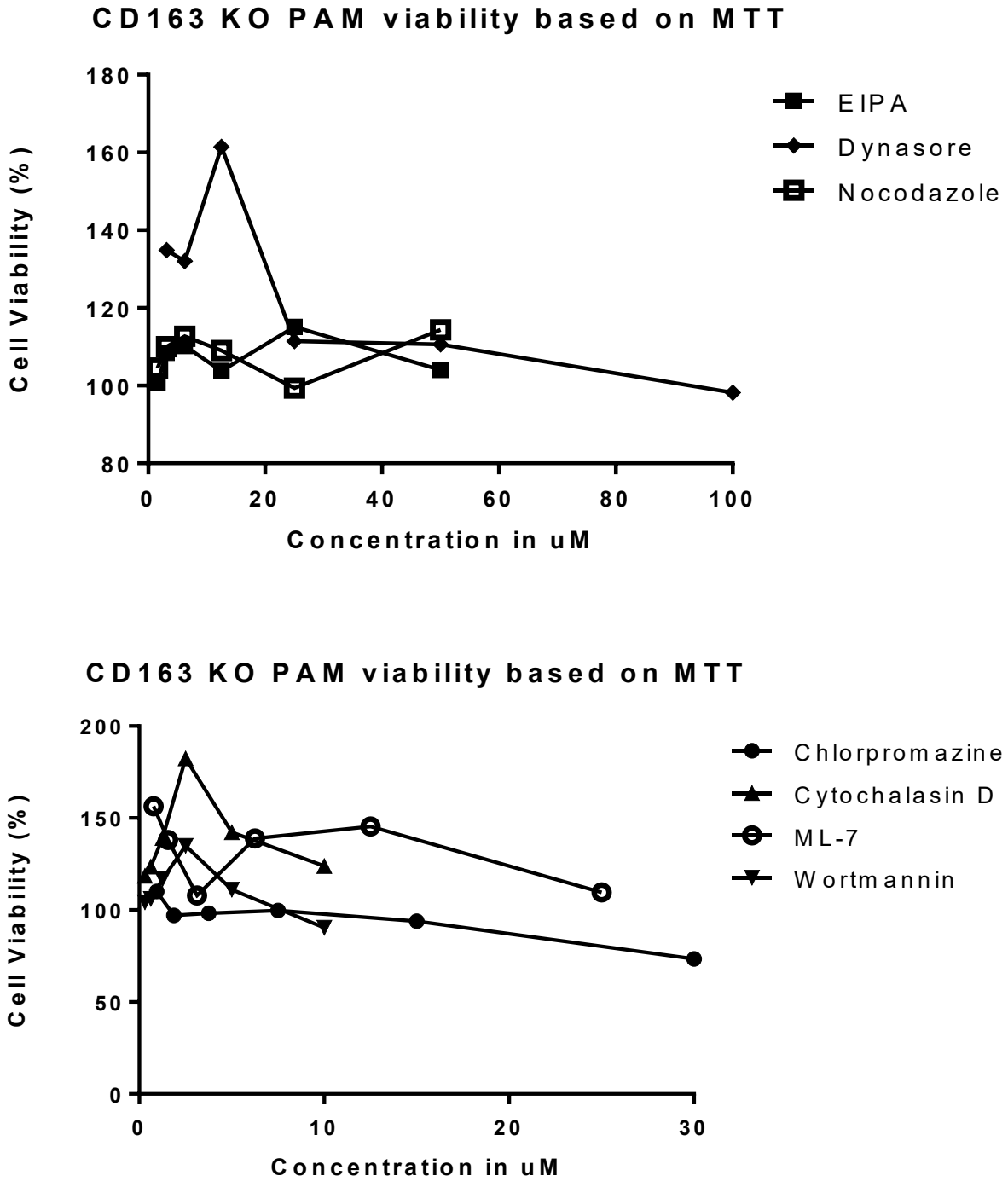


Table 3.1 Comparison of wildtype and CD163KO MTT results

Results were not found to be significantly different between the two cell types, suggesting the gene knockout does not play a role in cellular response and viability when treated.

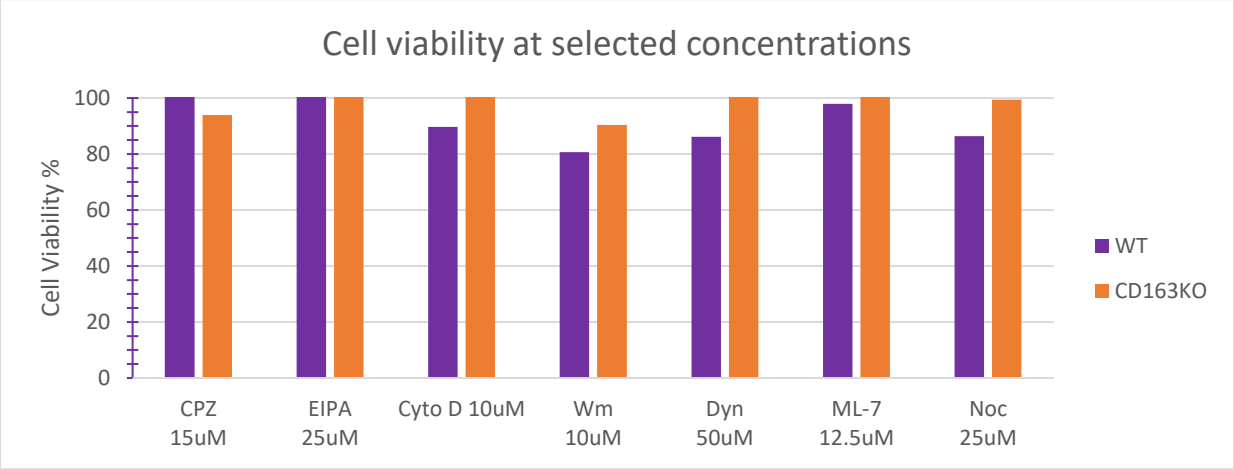


Figure 3.3 ASFV Georgia07 inhibition on WT PAMs

(* = p ≤ .05, ** = p ≤ .01, *** = p ≤ .001)

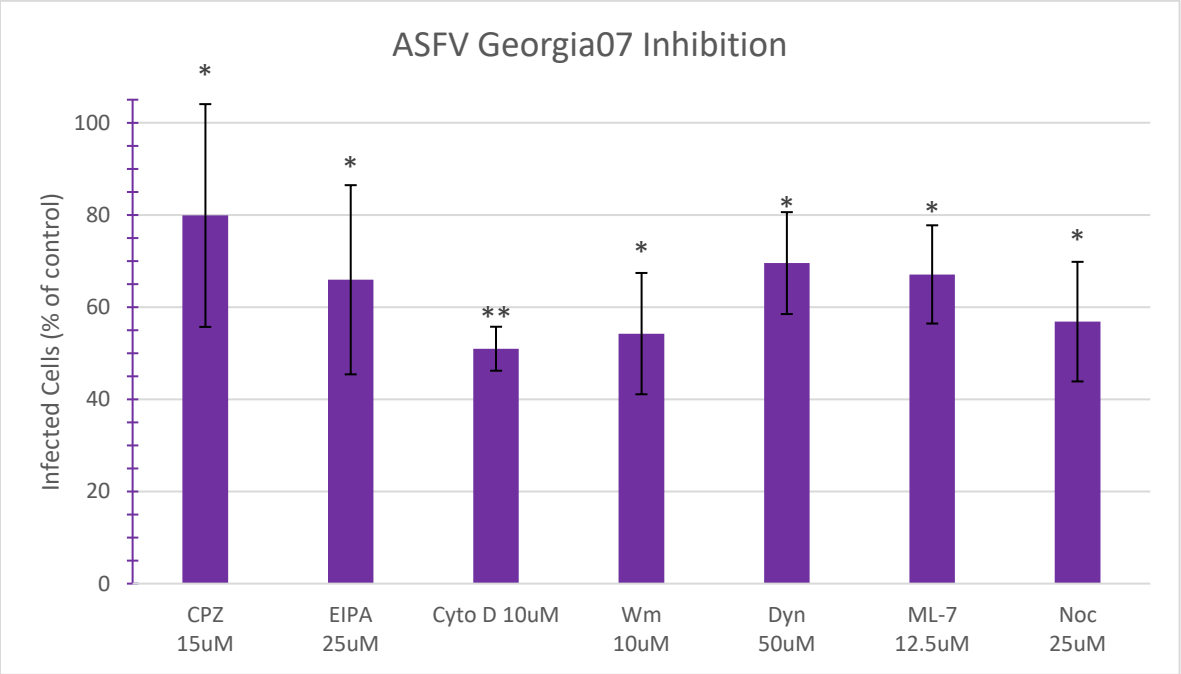


Figure 3.4 ASFV Georgia07 inhibition on CD163KO PAMs

(* = p ≤ .05, ** = p ≤ .01, *** = p ≤ .001)

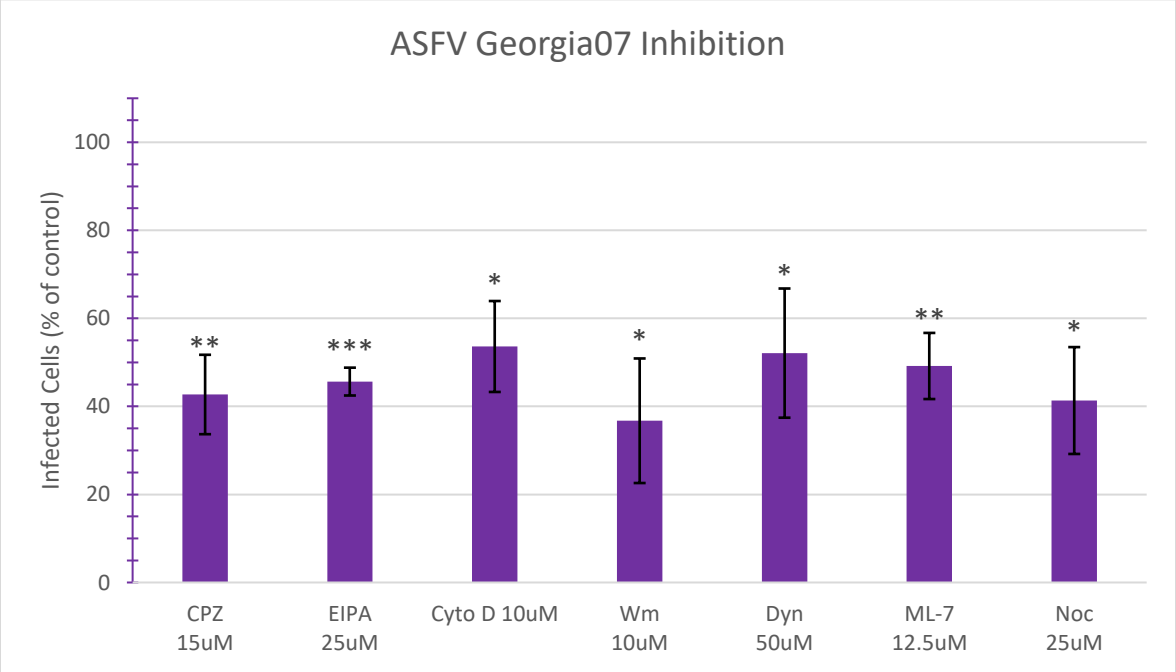


Figure 3.5 Wild type PAMs infected with ASFV Georgia07 stained with anti-p30 antibody

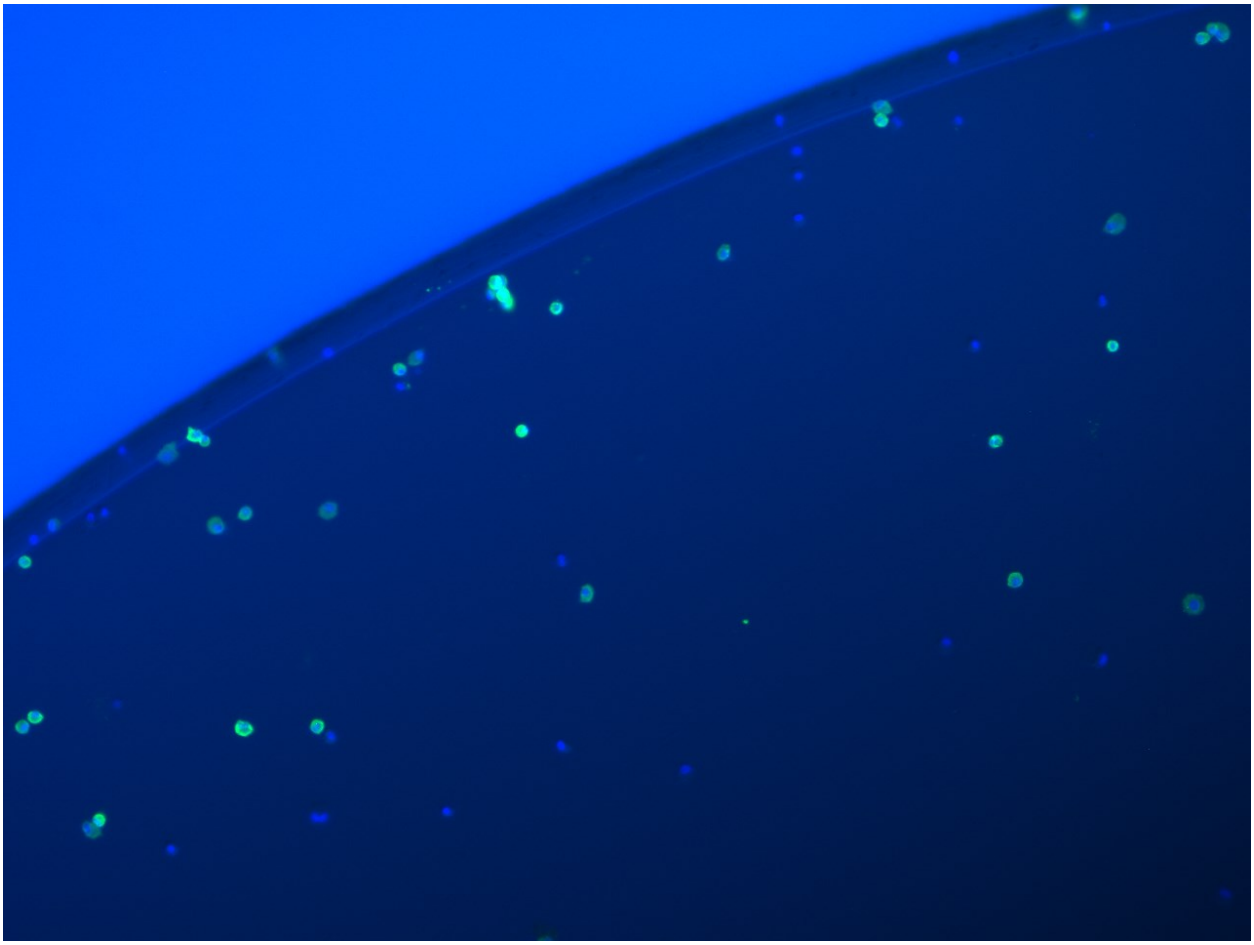
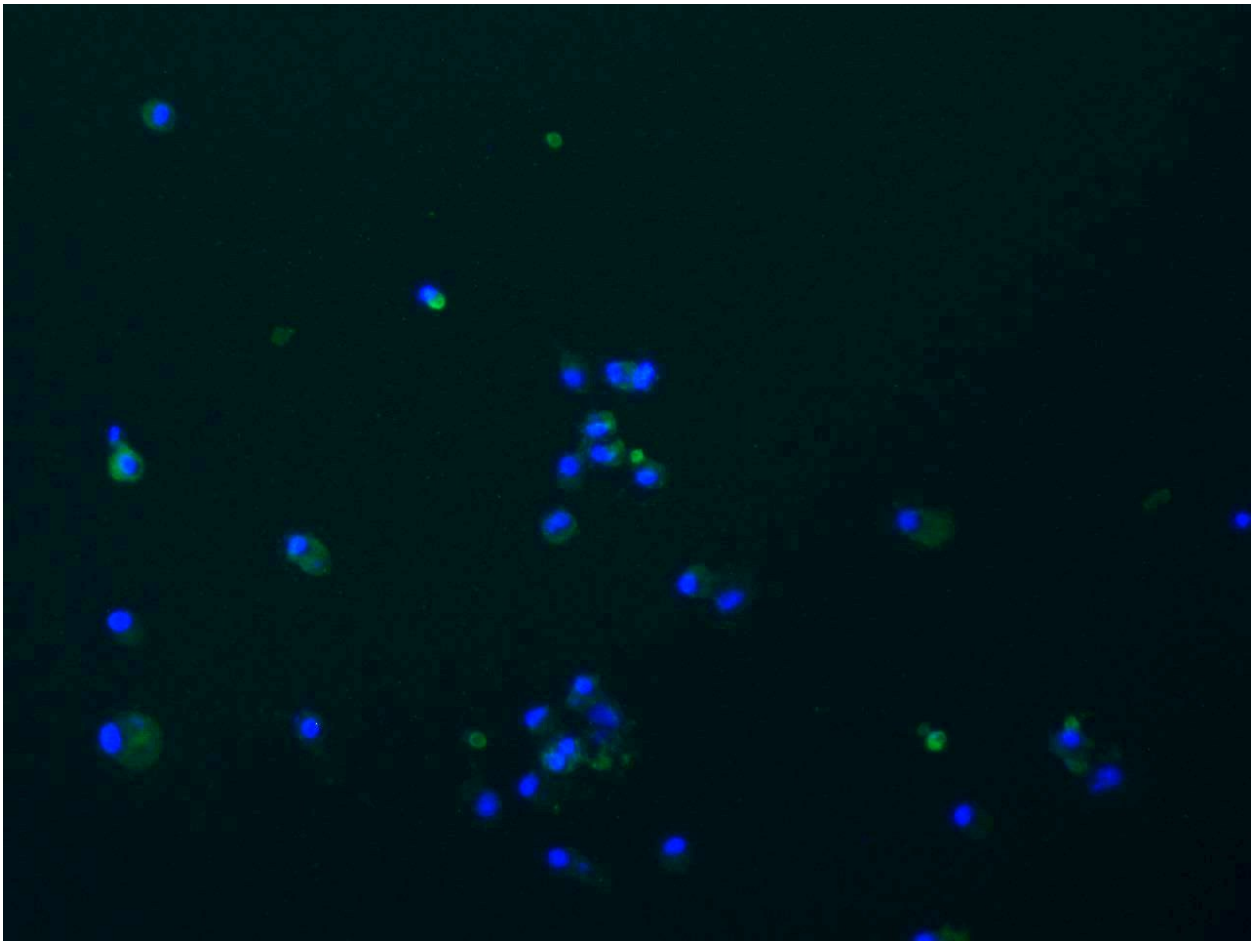


Figure 3.6 Wild type PAMs (uninfected) stained with anti-CD163 antibody



Chapter 4 - Concluding remarks

African swine fever virus currently presents the greatest risk to global swine health. Since the introduction of the disease into Georgia in 2007, the virus has seen unprecedented global spread and has affected many countries throughout Europe and Southeast Asia. As a result of the outbreak trade has been impacted and millions of animals have been destroyed, leading to significant economic impact. Transcontinental spread of the virus is believed to have occurred due to the movement of contaminated garbage and pork products, with wild boar contributing to the spread of disease in Eastern Europe.

Due to the complexity of the virus, there is still only a basic understanding of viral pathogenesis and host immunity. Two notable challenges of working with ASFV include the regulatory requirements of the virus, and the need for primary cells. Similarities between *Asfarviridae* and *Poxviridae* have led to Vaccinia virus to be identified as a useful surrogate for ASFV. However, VV has been little evaluated in Vero cells compared to other cell lines. While VV may be more sensitive to the disruption of clathrin- and dynamin-dependent endocytosis than BA71V, overall their response to the inhibitors suggests similar behavior during cell entry and early infection. Vaccinia may prove a useful surrogate for future studies, and can be used with both Vero cells and primary porcine macrophages.

While it is understood that ASFV uses macropinocytosis, the cause of ASFV's tropism is unknown. Clathrin-mediated endocytosis is also purported to be involved in cell entry, with CD163 being a proposed receptor. Studies have found contradictory results, though it is safe to say that CD163 is not required for infection. This work took that a step further to try to answer if CD163 is involved in infection at all. The CD163KO macrophages showed a similar response to

the inhibitors used, with all seven reducing the percent of infected cells relative to the untreated control. This corroborates that ASFV enters cells using both macropinocytosis and clathrin- and dynamin-dependent endocytosis, and utilizes non-muscle myosin II and microtubules during stages of entry and early infection. Ultimately, the lack of a significant difference in inhibition of virus infection between the wildtype and CD163 knockout macrophages suggests that CD163 has no involvement in ASFV infection. Unfortunately, this still leaves us with the pressing question: if not CD163 then what receptors, if any, are involved in ASFV infection? ASFV continues to remain an agricultural pathogen of high concern and a serious potential threat. Many aspects of ASFV's pathogenesis are not yet understood, which adds to the challenge of developing treatments or vaccines. Continued research efforts are necessary to determine receptors that are important to ASFV replication, and factors that regulate viral tropism.

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